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EDITORS

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ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life, and appeared simultaneously with Volumes IX, X, and XI.

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THE SALTING OUT OF GELATIN INTO TWO LIQUID LAYERS WITH SODIUM CHLORIDE AND OTHER SALTS.

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(From the Chemical Laboratories of Stanford University, Stanford University, California.)

(Accepted for publication, March 29, 1928.)

INTRODUCTION.

Hofmeister (1) and his pupils were the first to conduct extensive studies on the precipitation of proteins by salts. Since that time, the precipitation of proteins or, more particularly in this case, of gelatin has been the object of much study (2). However the fact that under certain conditions proteins may be salted out into two liquid layers seems to have been noted only by Pauli and Rona (3), Spiro (4), and Hardy (2). Pauli and Rona, while studying the effect of salts on the setting and melting points of gelatin solutions, found that if a gelatin solution was precipitated at 30°C. by the addition of a neutral salt and allowed to stand some hours at the same temperature, two liquid layers are formed, the upper containing but little gelatin, the lower being rich in gelatin and correspondingly more viscous. Spiro observed that both casein and gelatin precipitated by sodium sulfate formed liquid layers if allowed to stand in a warm thermostat. Hardy found that by increasing the temperature from 20° to 30°C. it was possible to have two liquid layers in equilibrium with each other in the system edestin, salt, and water. Analysis showed each of the layers contained all three constituents. Spiro further found that the analysis of the lower liquid layer closely agreed with that of the freshly precipitated protein. He points out the similarity between this phenomenon and the salting out of alcohol. In neither case is the phenomenon one of precipitation since "owing to the appropriation of water by the salt" separation into two liquid phases occurs. Each phase contains all the constituents and any alteration in the concentration of any one of the three constituents leads to a readjustment of

the composition and relative amounts of the two phases. Since with alcohol the effect of the electrolyte is not attributable to a specific action of the constituent ions, Spiro points out that such influence of the latter in the salting out of proteins must be regarded as subsidiary. This conception would explain the divergent results obtained in the salting out of proteins with neutral salts when the conditions are not maintained constant.

One source of confusion in the literature dealing with the precipitation of proteins is that there are really two distinct types of precipitation and many writers attempt to correlate data which depend on basically different phenomena. The one type is rightly termed coagulation or precipitation for it resembles the precipitation of suspensoid-hydrosols in that the colloid particles carry an electric charge due to the partial dissociation of their stabilising agent. With this type the precipitating agent is always decomposed or reacts with the stabilising agent. For it to occur, the proteins must be partially ionized, and precipitation is usually brought about by electrolytes of which only small amounts are necessary. The other type is best designated as salting out, for Spiro showed that it is analogous to the salting out of alcohol. Here the agent is not decomposed, the protein may be ionized or not and really large amounts of electrolyte are necessary.

Hardy (2) and Scaffidi (2) applied the phase rule to the systems serum globulin, salts, water; Galeotti (2) has applied it to the systems egg albumin, copper sulfate, water; serum albumin, copper sulfate, water; serum albumin, silver nitrate, water; egg albumin, sodium sulfate, water. Since each may consist of two immiscible components, protein and salt, which are partially miscible in a third component, water, they have been compared with the system succinic nitrile, sodium chloride, water studied by Schreinemakers (5).

Robertson (6) points out that Galeotti finds that solid $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, solid protein, and water can coexist, the water being saturated with $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and also containing dissolved protein, as is shown by the fact that dilution of the fluid phase causes further precipitation of protein. From this he argued that protein in solution was not to be regarded as a separate phase for otherwise he would have to postulate four coexistent phases. With regard to this point McBain and Burnett (7) have formulated the statement that for purposes of the

phase rule, a solution of a reversible colloid, no matter how complicated in itself, behaves towards external equilibria as a single phase.

Proteins are not the only colloids which can be salted out, for other substances in the colloidal state exhibit the same phenomena. In fact it is upon this that commercial soap manufacture depends and these phenomena have been far more extensively studied in the case of soaps than for any other class of substance. The results here detailed for gelatin bear a close resemblance to the behaviour of the corresponding solutions of soap as established by the comprehensive work of McBain and his collaborators (7).

EXPERIMENTAL.

Materials.

The salts used were for the most part Kahlbaum's best. Most of the work was done with Eastman ash-free gelatin, though best photographic gelatin (obtained from Dr. Slater Price, Director of the British Photographic Research Association), Coignet's silver label gelatin, and Coignet's gold label gelatin were also used. Analysis of the gelatins for water and ash showed:

Gelatin	Water	Ash
	<i>per cent</i>	<i>per cen</i>
Photographic.....	18.8	0.88
Coignet's gold label.....	15.4	1.36
Eastman ash-free (used mostly).....	11.3	0.08
“ “ “ (sample used in last few experiments).....	11.2	0.12

The conductivity of Eastman ash-free gelatin was approximately 10^{-4} mhos in 10 per cent solution indicated that the gelatin contained but very little electrolyte. The effect of any such salt present was negligible for the purpose of this investigation inasmuch as such large quantities of salt are afterwards added.

Hydrogen ion determinations were made with an electrode designed according to Considine (8). Considerable difficulty was met with in determining hydrogen ion, especially in 20 and 30 per cent solutions of gelatin, but results apparently accurate to 0.1 pH could be obtained by replatinizing the electrode after each determination.

Solutions of gelatin containing a given percentage of dry gelatin per 100 gm. of solution were made up as follows. The required amount of gelatin, cut in small pieces, was placed in a beaker with a few crystals of thymol (to prevent mould), covered with the requisite amount of water, and allowed to stand at least 3 hours. The swollen gelatin was placed in an oven and allowed to stand at 35°C. for at least 12 hours. To 100 gm. portions of the resulting solution in 150 cc. glass stoppered bottles varying amounts of salts in the solid form were added. The solution was frequently shaken and kept in the oven at 35°C. till the salt dissolved. Then the desired amount of acid was added and the solution allowed to settle out overnight into two layers. The following day the layers were thoroughly mixed and separation again allowed to take place. This is found necessary for, especially in critical regions, a labile lower layer may be set up on first addition of acid which later, on stirring well, redissolves. A similar effect has been noted by Höber (2). That is, less salt is required at first for production of two liquid layers. This change with time is characteristic of other properties of gelatin, especially osmotic pressure and viscosity. It is possible that the same influences, possibly a change in the amount of aggregation (9), play the same rôle here. Moeller (2) thought the change in amount of precipitated gelatin was due to hydrolysis which would naturally be greater the more the acidity. At any rate to obtain reproducible results, the effect of time must be allowed for. Another property which changes with time is the acidity which has been found to decrease gradually on standing. This alone might well account for decreased separability with time since with all the salts employed an increase in hydrogen ion results in an increase in the gelatin salted out.

After settling has again occurred the layers were separated and analyzed. The upper layer was partially decanted and complete removal facilitated by cooling the system down to 0°C. in an ice bath, whereupon the lower layer gels and the upper layer may be drained off. It is found that this cooling down does not appreciably affect the equilibrium already established, for owing to the high viscosity of the lower layer changes take place very slowly. (In fact a stable lower layer at 60°C. which will dissolve on shaking at 50°C. may be preserved several days without apparent alteration at

room temperature. This is a familiar phenomenon in viscous solutions of soap.) The lower layer was then weighed and both layers analyzed for hydrogen ion for loss at 110° , for loss on gentle ignition, and for residue. Since the amount of acid added is known, and the amount of free acid is known from the pH value, the amount of sorbed or combined acid may be calculated. The free acid is given off at 110° and the combined acid on ignition. Hence the loss at 110° may be corrected to give gelatin content. The residue is sodium chloride.

Qualitative experiments showed that the phenomenon of separation into two liquid layers was a perfectly reversible one which obeyed the phase rule in that a change in temperature, or any of the four components, gelatin, water, salt, or hydrogen ion, resulted in a change in composition and amount of the two layers. The lower layer is always darker in color (unbleached gelatin was used) and more viscous than the upper layer. The lower layer also contained less salt and water and more gelatin than did the upper. Depending upon the conditions the lower layer varied from a clear solution to the dense, opaque, plastic layer which developed when the gelatin in the lower layer was about 30 per cent. Complete separation of upper layer from the viscous lower layer is ordinarily difficult.

On warming up a clear lower layer under the microscope, the first change observed is a clouding of the field with the immediate appearance of many fine indefinite discontinuities. These increase slightly in size and then begin to branch out and intertwine with each other in an indefinite network. (A slight tendency toward spiral formation is noted.) This network increases in fineness and complexity so that the system seems almost granular. Then on cooling down, the first change is the vague formation of a "dried mud flat" appearance which later becomes more pronounced. The cracks are clear while the spaces which they surround exhibit the above mentioned network. On further cooling the cracks widen and the system is composed of droplets in which the network is gradually diminishing. The drops decrease slightly in size and suddenly disappear at a definite temperature. On heating and cooling again the same changes are observed. If before the droplets entirely vanish, the system is heated again, the drops do not change in size but the same changes as noted above go on both inside and outside the drops until finally their outlines become

TABLE I.

Amounts of Salt, or of Salt and the Acid with Common Ion, Added to 100 Gm. of Gelatin Solution, Showing Whether or Not Separation into Two Liquid Layers Occurs, and If So the Ratio of Volume of Upper to Lower Layer.

Gelatin	Salt	Homogeneous		Two liquid layers		
		Salt	Acid	Salt	Acid	Ratio
<i>per cent</i>		<i>gm.</i>		<i>gm.</i>		
9.0 silver label	KCl	20.0		27.0		
10.0 gold label	(NH ₄) ₂ SO ₄	10.0		10.0	7 drops*	
10.0 " "	"	10.0		18.0		0.87
10.0 " "	K ₂ SO ₄	10.0	0.2 cc.*	No separation		
10.0 " "	Na ₂ SO ₄	9.0		10.0		1.2
10.0 " "	"	9.0		9.5		0.6
10.0 " "	"	9.0		12.0		1.7
10.0 " "	"	9.0		13.0		2.0
17.6 photographic	"	9.0	0.2 "	10.0	0.4 cc.	
18.1 ash-free	"	9.6	0.6 "	9.6	1.0 "	
10.0 gold label	KNO ₃	30.0	11 drops*	35.0	11 drops*	4.1
10.0 " "	"	30.0	11 " *	35.0	24 " *	4.0
10.0 " "	NaNO ₃	30.0	5 " *	30.0	11 " *	3.0
10.0 " "	"	30.0	5 " *	30.0	24 " *	3.5
10.0 " "	NaCl	26.0		27.0		2.8
10.0 " "	"	10.0		10.0	6.0 " *	
10.0 " "	"	31.0		31.0	2.0 cc.	3.3
10.0 " "	"	19.0	5.0 cc.	20.0	5.0 "	5.5
10.0 " "	"	20.0	4.0 "	20.0	7.5 "	3.1
10.0 " "	"	15.0	6.5 "	15.0	7.0 "	20.0
10.0 " "	"	10.5	10.0 "	15.0	10.0 "	5.0
10.0 " "	"	11.0	7.5 "	11.0	8.5 "	5.0
10.0 " "	"	12.0	20.0 "	12.0	10.0 "	3.9
1.0 " "	"	27.0		20.0	1.0 "	53.0
20.0 " "	"	20.0	7.5 "	20.0	15.0 "	0.52

* Drops of concentrated acid, in all other cases a 1.0 N solution of acid was used.

indistinguishable. At the concentrations studied there is never any formation of any anisotropic phase and both layers are always isotropic.*

* An observation that could not be repeated was that when 50 cc. of a 10 per cent ash-free gelatin solution, to which 10 cc. of N HCl was added, was allowed to evaporate slowly at 35° to about 30 cc., distinct crystals separated out.

Sodium chloride, potassium chloride, sodium sulfate, ammonium sulfate, sodium nitrate, and potassium nitrate were all capable of salting out each of the gelatins employed, but the amounts and acidity required for this varied according to the characteristics of the gelatin. A high ash content in the gelatin seemed to be required for separation with sodium chloride, because on adding this salt, without acid, to 1.1 per cent solution of gelatin free from ash, no separation could be obtained at 35°, even when the solution was saturated with sodium chloride (24 per cent). However, the solution was quite cloudy and on increase of temperature separation did occur. The gelatins containing ash probably contain sulfates which assist the separation. Salting out with potassium sulfate was found impossible, probably owing to its limited solubility.

Table I gives the composition of various mixtures showing which remained homogeneous, and which separated into two liquid layers. It is apparent that the order of strength of anions is $\text{SO}_4 > \text{Cl} > \text{NO}_3$, which is in accord with the Hofmeister or lyophilic series $\text{SO}_4 > \text{Cl} > \text{C}_2\text{H}_3\text{O}_2 > \text{NO}_3 > \text{Br} > \text{I} > \text{SCN}$.

Many workers (Moeller, Fenn, Loeb and Loeb, Michaelis and Davidsohn, Pauli, and Posternak (2), Lewith, Bancroft, Bogue, Morner, Mines, Procter (10)) have noted that an acid solution aids the salting out of many proteins. However only a few (Chick and Martin, Hardy, Mellanby, and Sørensen and Höyrup (2)) have recognized that the hydrogen ion also constitutes a component, thus making a quaternary system composed of hydrogen ion, water, sodium chloride, and gelatin. The salts as well as the acid added change the pH (11), and since the relations between salt, acid, and gelatin are probably complex, the only satisfactory method of determining pH would be electrometrically. Unfortunately with most of the experiments carried out the pH was not closely enough determined to give an accurate picture of the distribution of the hydrogen ion between the upper and lower layers, as they do not differ markedly in pH. It was noted however that usually the more completely the gelatin was separated the greater was the hydrogen ion concentration of the upper layer as compared with the lower one. Also the presence of much salt seemed to diminish the hydrogen ion concentration of the upper layer relative to that of the lower. It apparently is possible to have either a system

TABLE II.
Analyses of Systems.

Total HCl in system	Composition of system*		Composition of upper layer*				Composition of lower layer*			
	NaCl	Gelatin	NaCl	Gelatin	pH	Weight of layer	NaCl	Gelatin	pH	Weight of layer
gm.	per cent	per cent	per cent	per cent		gm.	per cent	per cent		gm.
0.000	23.4	11.0	No separation. The solution is saturated with NaCl				On warming separation occurs			
0.051	20.8	7.5	Separation about to occur							
0.077	18.5	8.1	No separation							
0.077	19.9	9.9	Separation about to occur							
0.077†	20.0	8.0	19.9	6.7	4.1	116.3	17.7	17.4	4.1	8.7
0.077†	20.1	7.9	20.5	6.9		113.6				12.4
0.077			20.5	7.2	4.1	117.6			4.0	8.5
0.077	20.7	9.0	20.8	8.6		124.0				5.0
0.077	21.5	9.2	21.7	8.0	4.1	112.2	19.2	18.8	3.8	13.3
0.077			22.7	4.8	4.2	100.4			4.2	27.5
0.077			23.2	4.3	4.3	99.4			4.3	30.7
0.077	23.3	7.2	24.4	3.2		102.5	19.4	20.6		30.0
0.077	23.3	7.3	24.5	3.4	4.0	102.7	19.4	20.8	3.8	30.1
0.11	20.6	7.5	21.5	4.0	3.2	102.8	17.3	20.5		26.9
0.145	15.8	8.2	No separation							
0.145	16.3	7.9	No separation							
0.18	15.8	8.2	16.2	6.1	3.0	100.2	13.5	18.9		19.8
0.18	15.8	8.2	15.9	7.5	3.5	118.5	13.7	19.5	3.5	7.3
0.18	16.7	8.3	17.3	5.2	3.5	101.1	13.0	24.5	3.3	18.4
0.18	18.5	8.1	19.8	2.7		93.7	14.7	24.5		30.3
0.18	20.5	6.9	21.9	1.2	3.3	102.4	14.9	28.5	3.3	26.8
0.18			21.9	0.4	3.25	106.6				24.8
0.18	21.3	7.2	23.6	0.77		103.7	16.0	27.4		31.8
0.22†	13.1	8.7	13.2	7.9	3.3	106.2	11.0	19.4	3.1	7.9
	16.3	7.9	17.2	4.0	2.9	95.8	13.2	22.1		26.4
0.23	13.1	7.2	13.3	3.5	2.9	92.5	10.1	21.7	2.9	22.3
0.23	13.2	8.3	Separation about to occur							
0.27	15.8	8.0	17.25	1.5	3.2	94.6	11.3	27.5	3.15	31.1
0.27	16.1	8.2	17.5	1.6	3.0	94.2	11.4	28.9	2.9	29.7
0.27	20.2	7.4	22.1	0.64	3.2	102.0	13.6	30.9	3.1	29.5
0.29	10.6	7.9	Separation about to occur							
0.29	10.7	8.9	11.1	6.1	2.9	86.3	9.4	17.2	3.0	26.0
0.31	9.9	9.0	10.1	7.7	2.5	92.7	8.9	15.5	2.7	18.6
0.35	13.2	8.3	14.0	4.6	2.0	94.5	9.7	26.4	2.0	18.9

* Water constitutes the remainder of the system and may be calculated if desired by subtraction.

† If the system had been stirred, the analyzed layers would have mixed homogeneously.

TABLE II—*Concluded.*

Total HCl in system	Composition of system*		Composition of upper layer*				Composition of lower layer*			
	NaCl	Gelatin	NaCl	Gelatin	pH	Weight of layer	NaCl	Gelatin	pH	Weight of layer
gm.	per cent	per cent	per cent	per cent		gm.	per cent	per cent		gm.
0.37	8.4	8.4	No separation				9.7	15.4	2.0	24.4
0.37†	8.9	8.9	9.1	7.3	2.0	88.2				
0.37	9.4	8.9	No separation							
0.37	9.8	9.2	10.1	6.6		86.8				
0.37	10.6	7.9	11.2	4.3	2.0	90.6				
0.37	13.5	7.9	14.1	5.0	1.4	96.9				
0.37	16.7	8.4	18.5	0.59	1.3	90.5				
0.55	8.4	8.4	No separation							
0.55	9.4	8.9	No separation							
0.55	16.4	8.2	18.1	0.68	0.9	93.1				
0.77	10.4	8.6	No separation				10.9	32.6	1.1	28.7
0.77	12.5	8.9	13.1	6.5		103.5				
0.77	16.2	8.1	17.7	1.5		97.0				
0.77	20.6	8.0	22.25	1.4		107.7				
0.77	20.6	8.0	22.25	1.4		107.7				
0.037	17.0	0.87	17.1	0.29	2.1	115.6				
0.037	21.7	0.85	21.8	0.12	2.0	122.4				
0.55	13.1	17.4	15.4	5.25		38.5				
0.55	16.3	16.3	20.1	1.16		41.9				
							14.3	24.4		80.4

in which the lower layer has a greater concentration of hydrogen ion than the upper, or in which the two layers are the same, or in which the upper layer has the greater concentration of hydrogen ion.

The effect of temperature on the separation of proteins by salts has been found to be quite complicated (*cf.* Chick and Martin, Fenn, Galeotti, Guerrini, Scaffidi, Hardy, Hofmeister, and Spiro (2)) and Lewith (10). As Fenn points out, there is much confusion and the data in the literature are conflicting, some writers finding for a given protein a positive temperature coefficient, others a negative one, and still others a negligible one. This is what would be expected from an inspection of our phase rule diagram depending on what fraction was chosen for comparison. Actually in many cases there is a reversal of the temperature coefficient at a definite temperature which varies according to the amount of salt and hydrogen ion in the system.

When conditions are such that there is considerable gelatin in the upper layer (5 per cent), an increase in temperature markedly assists in salting out. When there is but a small amount of gelatin in the upper layer (1 per cent) both raising and lowering of temperature may

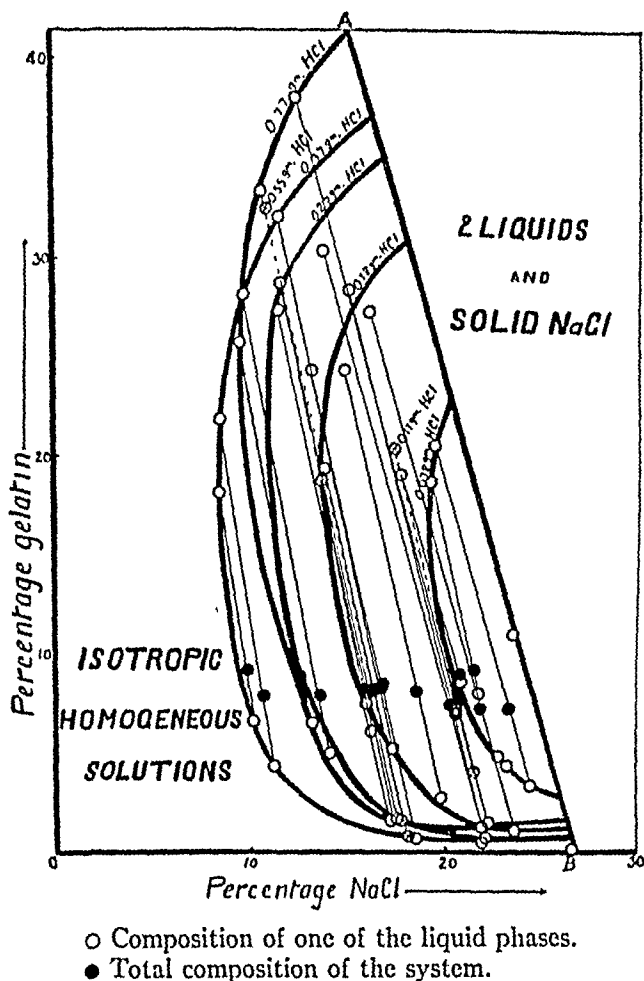


FIG. 1. Isotherms at 35° showing the salting out of aqueous gelatin by sodium chloride into two liquid layers, one isotherm for each concentration of hydrochloric acid. The region to the left represents insufficient salt for any separation; the middle region two liquid layers; and the region to the right excess of salt in solid form.

aid salting out. A further increase in temperature may even cause the solution of that separated at a moderate temperature. Undoubtedly the isotherms for various temperatures are not straight and

parallel with each other but exhibit maxima and minima and each other in a way like those found for globulin, water, and magnesium sulfate by Galeotti (2).

Table II gives the analysis of different systems studied using free gelatin, sodium chloride, and hydrochloric acid, and Fig. 1 shows

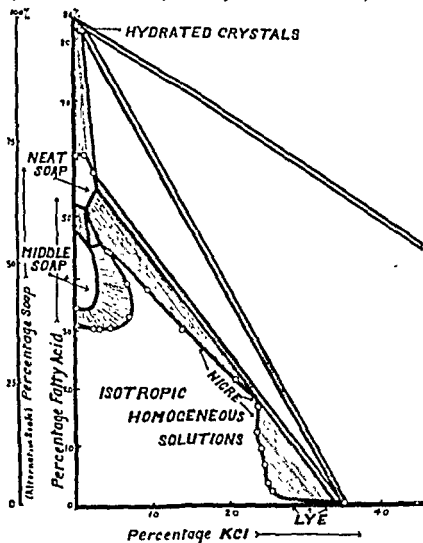


FIG. 2. Phase rule diagram at 90° , potassium laurate-potassium chloride-water; concentrations are expressed in grams of fatty anhydride and gram potassium chloride per 100 gm. of total system. The two liquid systems, neat lye, correspond to the areas in Figs. 1 and 3.

the isotherms at 35°C. of the systems containing the same amount of hydrochloric acid. The clear region on the left where little is present, represents a single isotropic phase. As the amount of is increased separation into two isotropic liquid phases occurs which would have nearly the same composition if the initial gelatin solution used was about 11 per cent. As more salt is added, the composition

and amount of the two layers varies until the solutions become saturated with salt. The solubility of sodium chloride in gelatin was determined only for an 11 per cent gelatin solution and it is assumed that the line AB, extended, to which all the isotherms are extrapolated, is a straight line. The experimental limits of error are indicated by the points which do not quite fall on the curves. Systems set up at the same time check very closely, but systems whose history varies show distinct discrepancies. Early work in which the systems were allowed to stand for the same time show the most marked differences. Most of the work, however, was carried out under the uniformly prescribed conditions already described. Possibly true equilibria are not fully reached but the smoothness and relative positions of the curves obtained indicate at least an approximation to it. Systems near the conditions necessary just to separate into two layers are undoubtedly the ones in which exact equilibrium is most difficult to obtain, and in these systems time of standing is a very important factor. It will be noted that all right-hand tie lines are approximately parallel to AB, the solubility curve of salt in gelatine and must be so; for if one liquid layer is just saturated with sodium chloride, the other must be so also. This, as well as the continuity of the curves and the parallelism exhibited by the tie lines, confirms the essential accuracy of the experimental data. To the right of AB lies the heterogeneous system composed of two liquid layers in equilibrium with AB, and crystals of salt. The curves for 2, 5, 7.5, and 10 cc. of normal acid form homologous series and the curve for 20 cc. acid indicates that a maximum for the separation of approximately 10 per cent gelatin solutions has been reached and passed. If sodium chloride were more soluble it is evident that no acid would be necessary for separation. Hydrochloric acid alone will not bring about the salting out, nor can the ash-free gelatin be salted out with sodium chloride in a solution rendered alkaline with sodium hydroxide.

The phase rule diagram for potassium laurate-potassium chloride-water at 90° found by McBain and Field (7) is shown in Fig. 2. The shape and location of the bay bounding the region wherein the two isotropic liquid phases, nigre and lye, coexist, strikingly resemble the bay found for gelatin-sodium chloride-water. It is of no great consequence that gelatin itself is not a definite chemical entity because

mixtures of soaps likewise yield phase rule diagrams which closely resemble those of single soaps (McBain (12)). Obviously since gelatin is so very like soap, as indicated by our diagrams, if anisotropic regions corresponding to neat soap and middle soap are possible

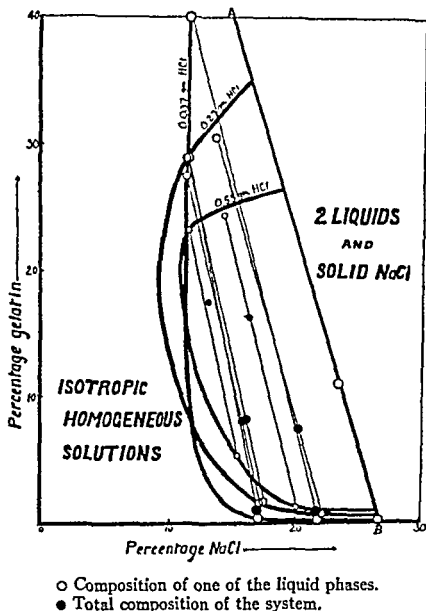


FIG. 3. Isotherms at 35° showing the salting out of aqueous gelatin by sodium chloride into two liquid layers, one isotherm for each concentration of hydrochloric acid. The total content of gelatin in the system is widely varied in these experiments.

for gelatin, they must be sought in regions where the concentration of gelatin is higher than 40 per cent.

Fig. 3 shows the differences obtained using widely different initial concentrations of gelatin. At first sight these curves might appear

to belie the applicability of the phase rule, since for any one upper or lower layer, the composition of the other ought to be fixed according to the phase rule regardless of the relative amounts of the two layers in equilibrium. However, this point cannot be tested fully by these particular data, because the acidity of the solution was not kept the same for all. Possibly, due to varying absorption or combination, the relations between gelatin, hydrochloric acid, and salt are not strictly comparable at different concentrations of gelatin. Chick and Martin (2) also found that the initial concentration of egg albumin influences its separation and though Sørensen (2) has criticized their data on the grounds that they did not allow for the alteration in ammonium sulfate content of the supernatant liquid, the differences are too large to be entirely accounted for in this way. By taking the effect of varying amounts of protein on the hydrogen ion concentration into account the whole behaviour can probably be accounted for in the way here presented.

SUMMARY.

1. Conditions under which gelatin may be salted out into two liquid layers at 35° were studied.

2. The equilibria governing the amounts and composition of the layers salted out with sodium chloride are found to accord with the requirements of the phase rule for the quaternary system gelatin-sodium chloride-hydrogen ion-water.

3. So far, soaps and gelatin are found to be surprisingly similar in their behaviour and definite indications are given as to where further similarities may be sought.

4. It is evident from this work that the term "coagulation" as ordinarily applied to the salting out of proteins is definitely a misnomer.

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HEMOLYSIS OF CHICKEN BLOOD.

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This paper is concerned with the kinetics of hemolysis of chicken red cells by the simple hemolysins, saponin, sodium taurocholate, and sodium oleate. The action of these lysins has been extensively studied on mammalian red cells, but quantitative investigations on the nucleated cell are wanting.

Methods.

The methods used in this work are almost identical with those described by Ponder (1, 2). They will be briefly sketched here.

The standard blood cell suspension is usually taken as a 2.5 per cent suspension of erythrocytes, twice washed in 0.85 per cent NaCl. In this work, however, a double strength suspension, 5 per cent of chicken blood cells, was found to give a more distinct end-point and was therefore used throughout. The suspension is always made up from freshly drawn oxalated blood, and used within a few hours after preparation. The estimation of the time taken for the lysis is carried out in a constant temperature water bath, made with glass sides; this permits the contents of the tubes to be seen by transmitted light without the removal of the tubes from their environment.

The white lined background of the bath permits accurate determinations of the end-point for non-nucleated cells. But with the nucleated chicken erythrocyte the sharp end-point as found in the hemolysis of human cells is not obtainable, the lack of sharpness being due to suspended nuclei and ghosts remaining after the hemolysis is complete. The ruled background thus proves of little value. This necessitates the establishment of a new end-point for complete hemolysis of the nucleated red cell. This end-point is naturally more arbitrary than the definite one found in the hemolysis of non-nucleated cells, and it is only with much practice that consistent values are obtained in our experiments. To aid in these determinations two tubes for comparison are placed in the bath; one tube contains a suspension of cells in the same dilution but without the lysin, the other tube contains an

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example of complete hemolysis of the nucleated blood. The temperature of the bath is kept at the constant temperature of 25°C.

A series of stock dilutions of saponin are required. These dilutions are labelled 1 in 10,000, 1 in 20,000 up to 1 in 70,000. Any intermediate dilutions can be made as required. To keep the amount of the hemolytic agent which comes in contact with the cells in round numbers, the following method is used. A known weight of saponin is dissolved in sufficient 0.85 per cent NaCl to make it 2.5 times the strength indicated. For example, the dilution labelled 1 in 10,000 is really 1 in 4,000 saponin. When 0.8 cc. of this solution is taken and to it is added 0.8 cc. of saline and finally 0.4 cc. of the blood suspension, the resulting dilution is 1 in 10,000 with a final volume of 2 cc. These saponin stock dilutions keep without loss of hemolytic power for about a week in well stoppered bottles and in a cool place.

The dilutions of taurocholate are prepared as follows. The sodium taurocholate after having been weighed in a stoppered weighing bottle is dissolved in a quantity of 0.8 per cent NaCl to make a 2.5 per cent solution. From this solution the other necessary dilutions are made as required. As Ponder points out, among other precautions necessary to observe when working with taurocholate, the solution must be used as soon as the dilutions are made, because upon standing even a short time the taurocholate undergoes a change in physical state and loses some of its hemolytic power.

The same method as outlined for taurocholate preparation is used in the making of the sodium oleate solution. The dilutions are made from the 2.5 per cent solution of oleate.

In this hemolytic work great care must be exercised that all glassware is clean. The cleansing is done in distilled water; the glass is then exposed to live steam and dried in a hot air oven.

To illustrate how a determination is made an example follows. Of the saponin solution marked 1 in 10,000, 0.8 cc. is placed in a small dry test-tube of clear white glass (1.5 cm. by 10 cm.). To this is added 0.8 cc. of 0.85 per cent NaCl solution; the tube is then placed in the water bath. In the same bath, there are placed a vessel containing the blood suspension to be added to the lysin, and a tube containing a graduated pipette immersed in 0.85 per cent NaCl solution. When the suspension and the dilution of the lysin have acquired the temperature of the bath, 0.4 cc. of the blood suspension is added with the warmed pipette. The time between the addition of the blood suspension and the completion of the hemolysis is noted with a stop-watch.

This procedure is carried out for the different dilutions of the lysin. A time-dilution curve (as shown in Fig. 1) is thus obtained by plotting the dilution of the lysin against the time taken by it for the completion of lysis of the quantity of suspension used.

The observed curve is then fitted with the expression,

$$t = \frac{1}{\kappa} \log \frac{c}{c - x} \quad (1)$$

in which t is the time in minutes required for the completion of the lysis, c the initial concentration of lysin in the system measured in milligrams, x the amount in milligrams of lysin used up in producing the lysis, and κ a constant.

To determine the value x , we find the asymptote where $t = \infty$ and $c = x$. This is found first by inspection and then by trial; the value of c corresponding to the asymptote gives the value of x , the constant κ is then found from the above expression. All these curves have been determined several times, but only a representative one for each lysin is given here.

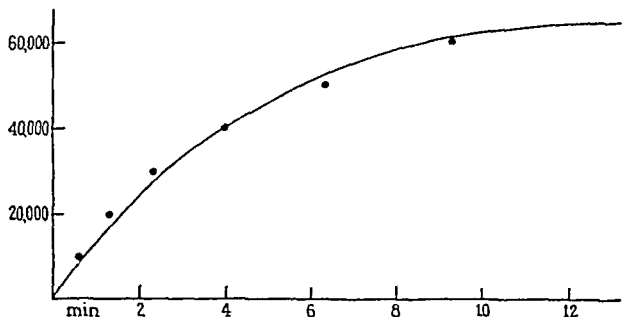


FIG. 1. Time-dilution curve of nucleated chicken red cell by saponin.

RESULTS.

1. *Hemolysis by Saponin.*—The relations between t , the time for complete hemolysis of the chicken cell suspension, and δ , the dilution of the saponin acting on the cells in the system of 25°C., is expressed below.

δ	t -observed	t -calculated
10,000	0.6	0.7
20,000	1.3	1.6
30,000	2.3	2.6
40,000	4.0	4.0
50,000	6.3	5.8
60,000	9.3	8.8
70,000	23.5	29.0

The values of t are calculated from the values $t = \infty$ and $\delta = 72,000$, hence $\alpha = 0.277$ and $\kappa = 0.203$. This calculated curve is given in Fig. 1, and the observed points plotted as small circles. From this graph it is obvious that the observed curve is described by the expression (1) with a goodly degree of accuracy; it may therefore be concluded that the time-dilution curve for the action of saponin on the nucleated red cell is very similar to the curve given by saponin on the non-nucleated erythrocyte.

2. *Hemolysis by Sodium Taurocholate*.—The time-dilution curve for the action of sodium taurocholate on the nucleated cell is given below. The notations are the same as under the action of saponin.

δ	t - observed	t - calculated
250	0.15	0.17
500	0.25	0.36
750	0.50	0.58
1,000	0.75	0.85
1,250	1.00	1.20
1,500	1.58	1.60
1,750	2.50	2.30
2,000	6.00	6.60

The values of t are calculated from $\alpha = 0.9091$ and $\kappa = 0.715$, where $t = \infty$ and δ is 2,200. It is found (Fig. 2) that the formula fits the observed curve. The curve is similar in almost every respect to that with the saponin, except in the range of effective dilutions. Whereas 1 in 72,000 of saponin is the dilution for hemolysis in infinite time, 1 in 2,200 of the taurocholate is necessary.

3. *Hemolysis by Sodium Oleate*.—In the action of sodium oleate on the nucleated chicken red cell a new point, not met in the case of the other lysins, must be considered. In high concentrations this lysin first attacks the corpuscular membrane, thus causing hemolysis as we have been considering it. The lysin then continues its action and destroys the nuclei and ghosts, and as a result a clear end-point, as in non-nucleated cell hemolysis, is reached. Thus from the one experiment two end-points for each dilution are found within a limited range of activity. From these two sets of data, two curves can be obtained, with a different value of κ and of $t = \infty$ for each. One curve is the

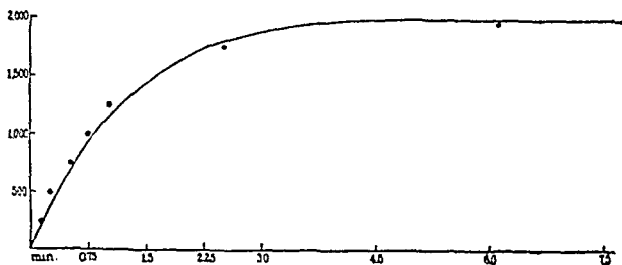


FIG. 2. Time-dilution curve of nucleated chicken red cell by sodium taurocholate.

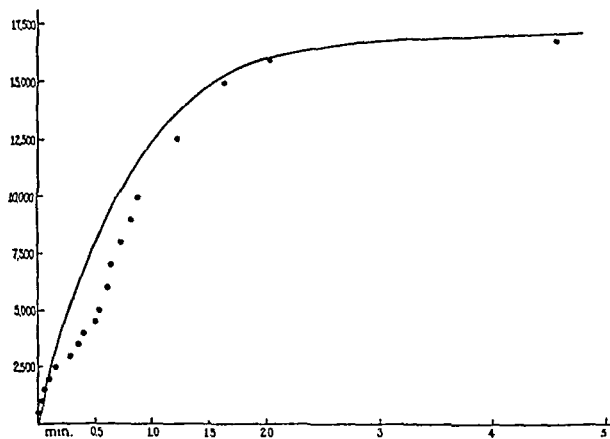


FIG. 3. Time-dilution curve of nucleated chicken red cell by sodium oleate. Hemolysis or cytolysis.

time-dilution curve for the action of the lysin on the corpuscular membrane, causing cytolysis, while the second is the time-dilution curve for the action of the oleate on the nuclear material, causing karyolysis.

Since the nuclei are destroyed only with very concentrated solutions of the oleate—in the range of 1 in 500 to 1 in 3,000—while, on the other hand, hemolysis occurs in dilutions up to 1 part of oleate in 17,500 parts of 0.85 per cent NaCl, the first curve is obviously less detailed than the second one. The two curves are given in Figs. 3 and 4.

The data for the hemolysis give:

δ	t -observed	t -calculated
500	0.02	0.02
1,000	0.03	0.05
1,500	0.07	0.07
2,000	0.10	0.10
2,500	0.16	0.13
3,000	0.28	0.15
3,500	0.35	
4,000	0.40	
4,500	0.50	
5,000	0.53	
6,000	0.60	
7,000	0.63	
8,000	0.71	
9,000	0.80	
10,000	0.87	0.67
12,500	1.20	1.00
15,000	1.60	1.50
16,000	2.00	1.90
17,000	5.00	4.50
17,500	8.50	7.80

The values of t are calculated from $\alpha = 0.1136$ and $\kappa = 1.23$. It can be seen from both the data and the curve (Fig. 3) that these observed values fit the theoretical curve (1) except between 1 in 2,500 and 1 in 10,000 where the rate of the hemolytic action is retarded. Why such an irregularity should occur cannot be definitely stated at this time. However, a somewhat similar case is found in the hemolysis of the non-nucleated red cell when sodium glycocholate is used (3).

The data for the curve (Fig. 4) formed from the clear end-points are:

δ	t -observed	t -calculated
500	0.10	0.29
1,000	0.67	0.69
1,500	1.16	1.16
2,000	2.00	1.82
2,500	3.25	2.81
3,000	9.30	9.40

The values of t are calculated from $x = 0.625$ and $\kappa = 0.541$. The fit of the observed values to the calculated curve is very good, and in the light of the difficulty in judging the end-point the curve is really

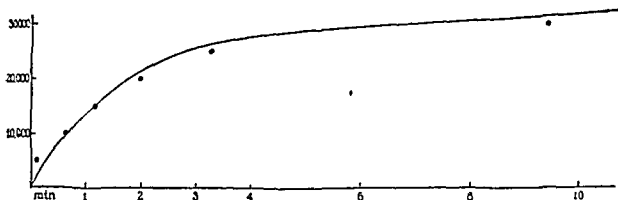


FIG. 4. Time-dilution curve of nucleated chicken red cell by sodium oleate. Karyolysis.

better than might be expected. This curve for karyolysis is of the first order and is of the same degree as the curve for the cytolytic action of the oleate.

After this peculiarity in the lower dilutions of the hemolysis curve with oleate was found, the taurocholate curve was investigated with a view to discovering whether any similar deflection occurs there. No such irregularity was found in this case. However, in the case of taurocholate, in a dilution around 1 in 2,000, the observed values for t do not always agree with the calculated one, but from time to time vary, with the result that this dilution gives a cluster of points rather than a constant one. This interesting phenomenon is under study at present.

Method of Determining Viscosity.

The determinations of viscosity are made by the Ostwald method. The system is placed in the constant temperature bath at 25°C. and with this method consecutive readings are made rapidly and without removing the fluid under observation from the bath.

The lysin, saline, and blood suspension are in the same concentration as used in the test-tube for determining the time-dilution curves of the lysin; *i.e.*, 2 cc. of the selected dilution of the lysin is put into the apparatus, then 2 cc. of 0.85 per cent NaCl is added. After these have reached the temperature of the bath, 1 cc. of the blood suspension is added and immediately the system sucked up into the calibrated bulb. The time consumed for the fluid to leave the bulb is recorded by a

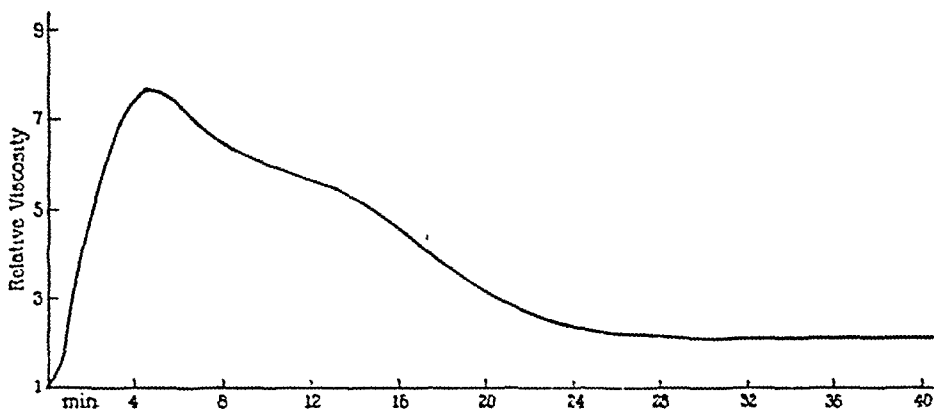


FIG. 5. Time-viscosity curve of sodium taurocholate-cell system.

stop-watch. These readings are continued until the viscosity becomes constant. The system is gently blown through prior to each reading to insure thorough mixing. The viscosity of water is taken as unity and the relative viscosities are found by comparison with that of water; the absolute viscosity at 25°C. is found by multiplying the relative viscosity by the constant 0.009. The time-viscosity curves are plotted with the relative viscosity on the ordinate and the moment of time at which the fluid is released from the top of the bulb on the abscissa.

For the sake of completeness, viscosity changes were looked for in connection with the hemolysis of the nucleated red cell by saponin, but none were found. The viscosity changes noted in connection with the hemolysis by taurocholate and oleate, respectively, occur only after the hemolysis of the cells is mostly, if not entirely, completed.

1. *Viscosity in Sodium Taurocholate Blood System.*—Viscosity changes with taurocholate are found with dilutions ranging between 1 in 100 and 1 in 600. It was found most convenient with our apparatus to take a medium strength solution, hence 1 in 400 is the strength of the sodium taurocholate used.

Although the curve is plotted from readings taken as frequently as possible, only part of the data is given.

Time	Relative viscosity	Absolute viscosity at 25°C.
min.		
0.3	1.21	0.0109
0.9	2.14	0.0193
1.7	4.86	0.0438
3.0	6.50	0.0575
4.6	7.36	0.0662
6.5	7.00	0.0630
8.0	6.50	0.0585
10.0	6.00	0.0540
13.0	5.50	0.0495
16.0	4.50	0.0405
19.5	3.00	0.0270
23.4	2.57	0.0231
25.9	2.29	0.0206
35.0	2.14	0.0193
60.0	2.14	0.0193
90.0	2.07	0.0186
120.0	2.07	0.0186

As can be seen from the figure (Fig. 5) the viscosity very rapidly reaches a maximum (7 or so times that of water) within 5 or 6 minutes after the blood suspension and the lysin have been mixed. The viscosity then drops, rather rapidly at first, then more slowly, until a point where the system is practically twice as viscous as water and there it remains.

2. *Viscosity with Sodium Oleate.*—The same type curve is found in respect to the sodium oleate and chicken cell suspension as is given by the action of sodium taurocholate. However, since the oleate has a stronger hemolytic activity than the taurocholate, a weaker concentration is used. A dilution of 1 in 1,200 is found to be about the best for this work.

HEMOLYSIS OF CHICKEN BLOOD

Time	Relative viscosity	Absolute viscosity at 25°C.
<i>min.</i>		
0.3	1.21	0.0109
0.9	2.14	0.0193
1.6	5.00	0.0450
3.0	9.64	0.0868
5.5	9.57	0.0861
8.0	8.64	0.0778
10.3	7.64	0.0688
14.3	7.14	0.0643
16.3	6.21	0.0559
17.9	5.64	0.0508
19.3	5.07	0.0456
20.9	4.36	0.0392
23.4	3.71	0.0334
26.5	3.21	0.0289
29.3	2.86	0.0253
32.6	2.57	0.0231
35.9	2.50	0.0225
70.0	2.50	0.0225

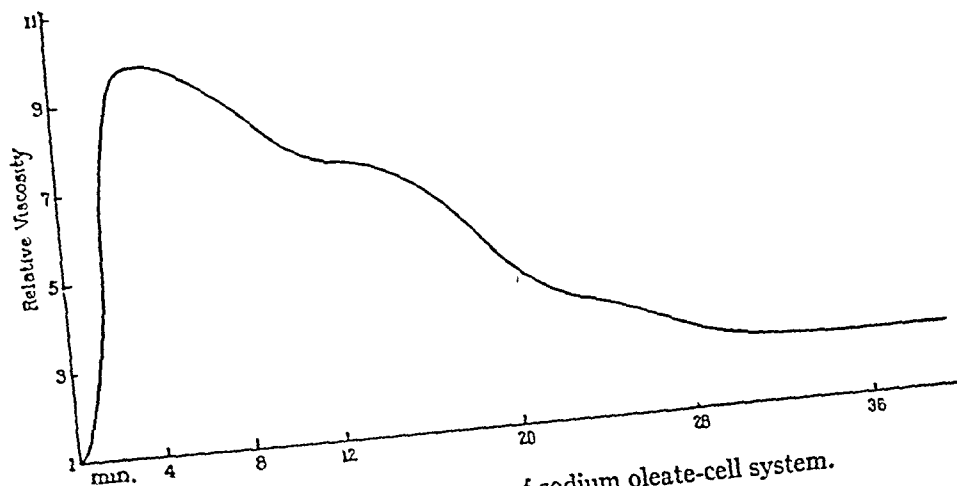


FIG. 6. Time-viscosity curve of sodium oleate-cell system.

In hemolysis by high concentrations of oleate, the nuclei are rapidly destroyed (karyolysis) before the viscosity change appears and the red fluid is transparent. However, within 10 minutes after hemolysis the fluid becomes cloudy once more.

As can be seen from the curve (Fig. 6) the system becomes almost 10 times as viscous as water after about 5 minutes. Then, as in the case of taurocholate, the curve gradually descends and in this case reaches a minimum viscosity after about 35 minutes. By a comparison of the oleate and the taurocholate curves, although the oleate solution is one-third the strength of the taurocholate solution, it will be seen that they are of the same general form and doubtlessly represent the same type of phenomenon.

The viscosity changes, both in case of oleate and taurocholate, do not occur until after the hemolysis of the nucleated chicken red cell is complete. The fact that taurocholate and oleate form loose compounds with the hemoglobin of the cells may have some relation to these viscosity changes.

Microscopic Action of Lysins.

Although no actual measurements of the changes in the shape, size, etc., of the nucleated erythrocytes were made during the action of the lysins upon them, the cells were frequently examined microscopically.

The chicken erythrocyte in 0.85 per cent NaCl appears ellipsoidal in flat view, except that each end of the cell is distinctly pointed. The nucleus appears to be a biconvex body in the center of the cell. After the lysin is added to the cell, it gradually loses its pointed ends and its ellipsoidal shape and becomes circular in flat view. After assuming this spherical shape, it gradually fades from view, leaving what appears to be a smaller nucleus in the center of a spherical ghost-cell. These observations hold for all three lysins used, and point to the fact that the mechanism of the hemolysis of the nucleated blood cell is similar to that found in the hemolysis of the non-nucleated mammalian erythrocyte (4).

SUMMARY AND CONCLUSIONS.

1. The time-dilution curves are given for the hemolytic action of saponin, sodium taurocholate, and sodium oleate on nucleated chicken erythrocytes.

2. Saponin and sodium taurocholate cause hemolysis but leave the nuclei and ghosts in suspension, thereby making the end-point of

hemolysis more arbitrary than the clear end-point for non-nucleated cell hemolysis.

3. The curves of hemolysis by saponin and taurocholate are shown to be of the same nature as are found in the hemolysis of non-nucleated cells.

4. Sodium oleate causes first hemolysis and then, in the stronger solutions, causes karyolysis. Two pairs of values for κ and $c = \infty$ are thus obtainable for the same reaction, one pair for the destruction of corpuscular membrane, the other pair for the destruction of the nucleus.

5. Viscosity changes are found in the lysin-cell system with strong concentrations of sodium taurocholate and sodium oleate. Time-viscosity curves are given for these changes.

6. Microscopically, the action of these lysins on the nucleated chicken red cell appears to be similar to their action on the non-nucleated erythrocytes.

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ELECTRIC IMPEDANCE OF SUSPENSIONS OF SPHERES.

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I.

The electric resistance of a suspension of homogeneous spheres is given by the relation

$$\frac{r_1/r - 1}{r_1/r + 2} = \rho \frac{r_1/r_2 - 1^1}{r_1/r_2 + 2} \quad (1)$$

in which r , r_1 , and r_2 are the resistances² of the suspension, the suspending, and suspended phases respectively, and ρ is the volume concentration of the suspended phase.

Since emulsions, suspensions of living cells, and colloidal particles have an interfacial surface layer which is markedly different from both the interior and exterior phases, the suspended phase can only be considered homogeneous under a few special conditions. As has been shown by Maxwell (9), §313, a sphere of radius a_2 and resistance r_2 surrounded by a concentric spherical shell of internal radius a_1 ,

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¹ This equation has been variously derived in the early theory of several physical problems but it often disagrees with the data (Lowry (8)) for atomic and molecular phenomena—probably because the assumptions underlying it are not valid in these cases. It has, however, been found by Fricke and Morse (3) to apply accurately to the resistance of cream up to 62 per cent volume concentration of butter fat, and its use is probably justified for the resistance of suspensions of spheres which are of much larger than atomic dimensions. A simple derivation is given by Maxwell (9), §314.

² Unless otherwise stated, all resistances, reactances, and impedances are specific, *i.e.*, for a centimeter cube.

external radius a_3 , and resistance r_3 , Fig. 1, can be replaced by a homogeneous sphere of radius a_3 and equivalent resistance \hat{r}_2 where

$$\hat{r}_2 = r_3 \frac{(2r_2 + r_3) a_3^3 + (r_2 - r_3) a_2^3}{(2r_2 + r_3) a_3^3 - 2(r_2 - r_3) a_2^3}.$$

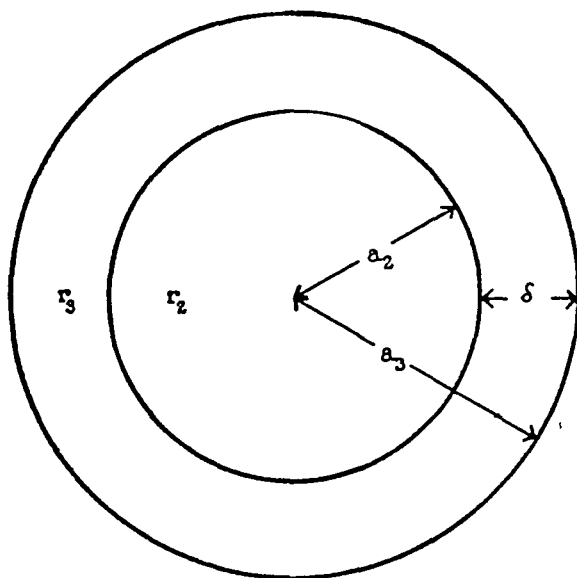


FIG. 1.

When $a_2 = a_3 - \delta = a - \delta$, where δ is a small quantity such that higher powers than the first can be neglected,

$$\hat{r}_2 = \frac{r_2 + \frac{\delta r_3}{a} \left(1 - \frac{r_2}{r_3}\right)}{1 - \frac{2\delta}{a} \left(1 - \frac{r_2}{r_3}\right)}.$$

If further, r_2/r_3 is small compared with unity,

$$\hat{r}_2 = r_2 + \frac{\delta r_3}{a}. \quad (2)$$

Assuming that the current flow in the surface layer is radial, then δr_3 , which is a resistance per unit area, may be replaced by z_3 , a complex

impedance per unit area given in alternating current vector notation (1, 7, 11) by

$$z_1 = r_1 + jx_1 \quad (3)$$

where the resistance per unit area r_1 is in series with a reactance per unit area x_1 and j is the imaginary operator. Substituting z_1 of Eq. (3) for δr_1 in Eq. (2) and replacing \hat{r}_2 by \hat{z}_2 the equivalent complex impedance of the sphere, we have

$$\hat{z}_1 = r_1 + z_1/a = r_1 + r_1/a + jx_1/a. \quad (4)$$

Solving Eq. (1) for r ,

$$r = r_1 \frac{(1 - \rho) r_1 + (2 + \rho) r_2}{(1 + 2\rho) r_1 + 2(1 - \rho) r_2}.$$

Substituting \hat{z}_2 for r_2 and replacing r by z , the complex impedance of the suspension,

$$z = r_1 \frac{(1 - \rho) r_1 + (2 + \rho) (r_1 + r_2/a) + (2 + \rho) jx_2/a}{(1 + 2\rho) r_1 + 2(1 - \rho) (r_1 + r_2/a) + 2(1 - \rho) jx_2/a}. \quad (5)$$

On the assumption that r_1 and r_2 are constant, when $z_2 \rightarrow \infty$, z is a pure resistance

$$r_0 = r_1 \frac{2 + \rho}{2(1 - \rho)}, \quad (6)$$

and when $z_2 \rightarrow 0$, z is again a pure resistance

$$r_\infty = r_1 \frac{(1 - \rho) r_1 + (2 + \rho) r_2}{(1 + 2\rho) r_1 + 2(1 - \rho) r_2}. \quad (7)$$

These are two cases in which the spheres may be considered homogeneous.

Solving Eq. (5) for z_2

$$z_2 = \frac{z - r_\infty}{r_0 - z} \gamma \quad (8)$$

where

$$\gamma = r_2 a \left[1 + \frac{(1 + 2\rho)}{2(1 - \rho)} \cdot \frac{r_1}{r_2} \right]. \quad (9)$$

When $r_2 = 3r_1$, and ρ varies from 0.2 to 0.4, the second term in the brackets varies from 0.3 to 0.5. Thus γ is of the same order of magnitude as $r_2 a$ and more or less constant for many cases.

Separating z into its resistance and reactance components,

$$z = r + jx,$$

the absolute value or magnitude of $|z_3|$ is given by

$$|z_3|^2 = \frac{(r - r_\infty)^2 + x^2}{(r_0 - r)^2 + x^2} \gamma^2. \quad (10)$$

In the special case where r_3 is constant or zero, it can be shown that

$$(r - r_\infty)(r_0 - r) = x^2.$$

Then

$$\frac{x_3^2}{\gamma^2} = \frac{r - r_\infty}{r_0 - r}; \quad (11)$$

and also

$$\frac{x_3^2}{\gamma^2} = \frac{|z|^2 - r_\infty^2}{r_0^2 - |z|^2}. \quad (12)$$

II.

When r_2 is finite and z_3 varies with the frequency n of the measuring current so that when $n \rightarrow 0$, $z_3 \rightarrow \infty$, and when $n \rightarrow \infty$, $z_3 \rightarrow 0$, then $|z|$ from Eq. (5) as a function of n is shown in Fig. 2.

If r_3 varies such that

$$r_3 = m x_3 \quad (13)$$

then it can be shown that as x_3 varies from 0 to ∞ the locus of the end of the impedance vector when plotted on the complex plane is an arc of a circle as shown in Fig. 3. Also the ratio of the chords a and b is

$$\frac{a}{b} = \frac{|z_3|}{\gamma}. \quad (14)$$

At the points r_∞ and r_0 , the slopes of the tangents are respectively $1/m$ and $-1/m$.

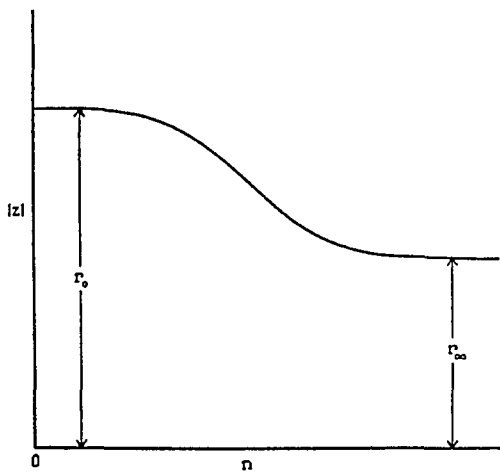


FIG. 2.

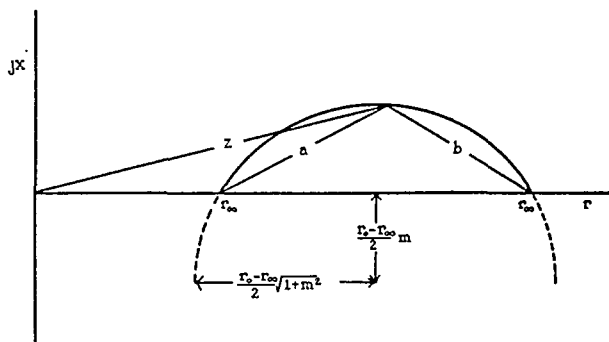


FIG. 3.

The case where $m = 0$, which gives a circle with its center on the resistance axis, has been treated in the first part of a paper by Carter (2). It will be noted also that his Eq. (10), for the case of a single reactance in a resistance network, is, as it should be, the exact counterpart of Eq. (5), above, when r_2 is constant.

III.

Zobel (13) has shown that certain types of two terminal networks—of which the circuits of Fig. 4 are special cases—can be made equivalent both in impedance and in phase angle for all frequencies. As a result

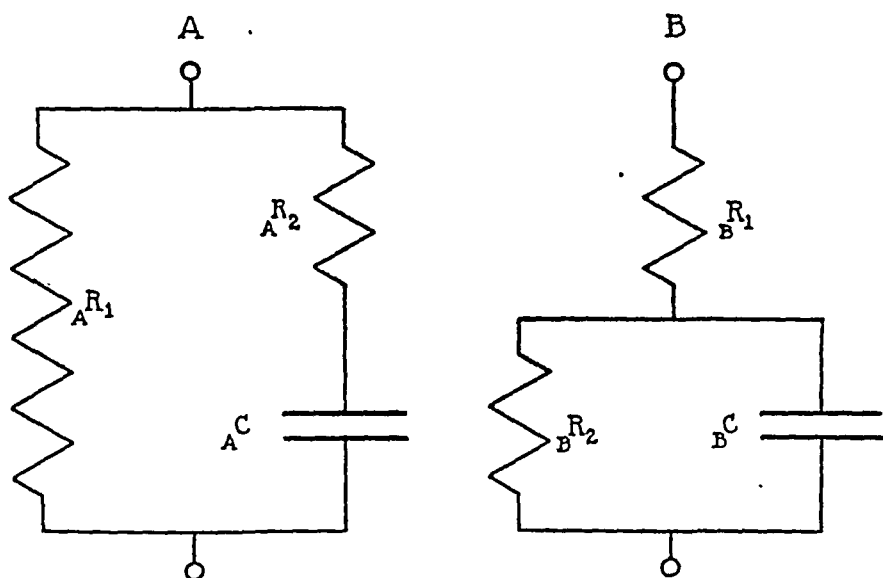


FIG. 4.

of this, such circuits containing any number of resistances and a capacity, can be made equivalent to either one of the two simple circuits. Thus it is evident that the number, location, and magnitude of the elements of such a circuit cannot be determined solely by electrical measurements made at the terminals, and that the number of circuits which can be made to fit a given set of data is probably limited only by the patience and ingenuity of the computer.

Fricke and Morse (6) found that their measurements of the resistance and capacity of suspensions of red blood cells at various frequen-

cies could be accurately fitted to a circuit of type *A*, where they thought of ${}_A R_1$ as due to the suspending medium, ${}_A R_2$ to the interiors of the corpuscles, and ${}_A C$ to the capacities at their surfaces. The values of these three quantities can be found for a suspension of spheres from Eq. (5) when $r_3 = 0$ and $x_3 = -\frac{1}{c_3 \omega}$, ω being 2π times the frequency in cycles per second. In this case ${}_A R_1$ depends on r_1 , and ρ , ${}_A R_2$ on r_1 , as well as on r_2 and ρ , while

$${}_A C = \frac{1}{2} (2 + r_1/r_0) (1 - r_1/r_0) a c_2.$$

For small volume concentrations this becomes approximately

$${}_A C \approx \frac{3}{2} (1 - r_1/r_0) a c_2, \quad (15)$$

which is the expression derived by Fricke (4) for spheres by a quite different analysis. Circuits of type *B* have been computed which fit the data, but they have no particular interest since Eq. (15) was derived on the basis of type *A*.

In his work on the impedance of tissues Philipppson (10) assumed a circuit of type *B* where ${}_B R_2$ and ${}_B C$ represented the resistances and capacities of the physiological cell membranes, and ${}_B R_1$ the resistances of the protoplasm of the cells in a centimeter cube of tissue. The data have been used equally well to compute circuits of type *A*, in which ${}_A R_1$ might be thought to be due to the intercellular electrolytes, ${}_A C$ to the membrane capacities, while ${}_A R_2$ involves the resistances of the protoplasm. Thus the interpretation of these data should not be made intuitively.

Whereas Fricke (5) found the red blood cell membrane to have a static capacity (*i.e.* independent of frequency), Philipppson (10) found a capacity which for animal tissues varied about as the inverse square root of the frequency, and for vegetable tissues as the inverse fourth root. Although unable to measure the magnitude of these capacities for a single membrane, Philipppson classed them as polarization capacities similar to those found at the surface of contact between metal electrodes and electrolytes. Such physical systems have a polarization resistance such that the phase angle, ϕ_3 , of the

combination is more or less independent of frequency and often in the neighborhood of 45° (see Wolff (12)). Then in Eq. (13), $\phi_3 = \epsilon \ln^{-1} m$. On the assumption that this angle was 45° , Philipponson calculated the series polarization resistance although he took no account of it in the computations of the capacities from the data. In Eq. (5), the polarization resistance would be represented by r_3 as a series resistance per unit area. Since r_3 would be a function of frequency, more or less proportional to α_3 , it would be difficult if not impossible to compute it from impedance data alone without making assumptions which are at present unwarranted. If, however, measurements are made of both the resistance and reactance of a suspension—with a capacity bridge, for instance—it becomes possible to determine both r_3 and α_3 from Eq. (5).

SUMMARY.

A general expression has been derived for the electric impedance of a suspension of spheres each having a homogeneous non-reactive interior and a thin surface layer with both resistance and reactance. The applications and limitations of impedance measurements on such suspensions are discussed.

The author very much appreciates the interest and assistance of Mr. K. S. Johnson of the Bell Telephone Laboratories, Inc., and of Professor E. L. Chaffee and Professor W. J. Crozier.

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ELECTRIC IMPEDANCE OF SUSPENSIONS OF ARBACIA EGGS.

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In work done several years ago on the heat production of the *Arbacia* egg at fertilization and during early development (11) the suggestion was made that the phenomena observed might be due to changes in the cortex. Inasmuch as the heat data can give no clue to the action of component parts of the egg, another means of analysis was sought which might give an answer to the various questions raised. The work of Fricke and Morse (3) and Philippon (10) suggested that the impedance of suspensions of eggs to various frequencies of alternating current might yield valuable facts regarding the electric capacity and resistance of the surface of the egg, and the resistance of the interior, and the changes of these three quantities at fertilization and during development.

Apparatus.

Although it was first planned to use a capacity bridge to measure both the resistance and the capacity of the suspensions of *Arbacia* eggs it was found after considerable work that the bridge had several very serious defects for this particular work. In the first place it became very complex as the range of frequencies to be investigated was extended (3). This made it necessary to devote an undue amount of time to the taking of each reading and the changing from one frequency to another. In the second place, unless one were willing to go to the very ultimate in amplification it was necessary to use an undesirably high current density in the electrolytic cell. In this work the aim was to keep the potential difference across each egg so low that the

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current probably would in no way affect it. From these and other considerations it was decided to use a variation of the "ammeter-voltmeter" method of measuring resistances. This method, while less sensitive and giving less data, is on the other hand direct reading and can be made independent of the frequency over a wide range. At first glance it would appear that this was the method used by Philippson (9), but it soon becomes evident that what he used was essentially a comparison method which involved many of the inaccuracies and troubles of the bridge method and gave only the results and accuracy of the direct reading method which has been used in the present work. A small alternating current of the desired frequency was sent through an electrolytic cell containing the suspension and simultaneous measurements were made of the current through the cell and the potential difference across it. The ratio of these two gave the magnitude of the impedance of the cell at that frequency but did not, however, give the phase angle.

Oscillator.—The source of supply of alternating current was a vacuum tube oscillator. This oscillator is of the type known as "tuned plate circuit" and while the type is well known (2) and very commonly used, this particular instrument has several unusual features of design and operation. Although its range can probably be extended in both directions, it has been used entirely between the frequencies of one thousand and fifteen million cycles per second. It is completely shielded electrostatically and is controlled only by switches on the panel over the entire range of frequencies. It has a readily changed but constant output and its frequency calibration has remained remarkably constant. While it is usual to take the output from such an oscillator by means of a coil loosely coupled to the tuned circuit, it was not found advantageous to do so in this oscillator. In addition to the necessity of switching both the grid and plate coils as the frequency was changed this output coil would have to be changed and it would also be a decided advantage in such a case to be able to vary the coupling. These needs would have made the oscillator so much slower and less flexible that it was decided to take the output from the main oscillatory circuit as is shown in Fig. 1. The inductance L' is very small as compared with the main oscillator inductance L but carries the oscillatory current flowing through the condenser.

Over the frequency range of any single inductance L the potential difference across L' remains comparatively constant. This output is connected to the electrolytic cell through a resistance sufficiently high to prevent reactions of the output back on the oscillatory circuit.

Frequency.—A two-turn coil was loosely coupled to the coil L'

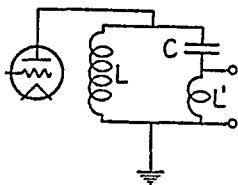


FIG. 1.

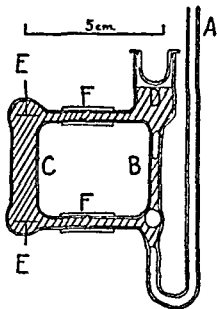


FIG. 2.

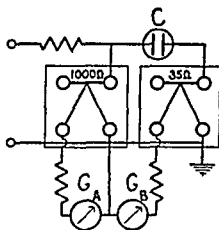


FIG. 3.

FIG. 1. Method of taking output from vacuum tube oscillator.

FIG. 2. Electrolytic cell and stirrer.

FIG. 3. Circuit for measurement of impedance.

within the oscillator. When the frequency was to be measured, connections from this coil were made through the shielding to a similar coil which was coupled to a General Radio Precision Wavemeter. The resonance point of the wavemeter was determined by means of a vacuum tube voltmeter connected directly across the variable condenser.

Electrolytic Cell.—The factors governing the design of the cell were several from both the biological and the electrical standpoints. In order to prevent settling of the suspended eggs it was necessary to have continuous but gentle stirring. It was also necessary to provide for adequate oxygenation of the suspension. The total volume of the cell should be small in order that a sufficient volume concentration may be obtained with the eggs from a single specimen. From the electrical standpoint it was desirable to have the cell constant such that the impedance would be a convenient value. It was also necessary to have the design such that at low frequencies the error in the impedance caused by the polarization capacity should be small while at high frequencies the error in the impedance caused by the static capacity should be small. For the first case, a comparatively simple analysis assuming a straight line current flow shows that for any given frequency, specific resistance of electrolyte, and polarization capacity per unit area of electrode, the error in the impedance is independent of the area of the electrodes but varies inversely as the square of their separation. On the other hand, a somewhat similar analysis of the error introduced by the static capacity shows that it is entirely uninfluenced by the shape of the cell—as long as the lines of current flow are straight—but is proportional to the product of the specific resistance and the frequency. For any given suspension the cell constant is fixed by the measuring apparatus and the volume of the cell is determined by the amount of material available. These two quantities then determine the dimensions of the cell and it remains to be seen if the error introduced by polarization capacity at low frequencies is negligible. If it is not then the apparatus must be modified, other material used, or corrections introduced. If the error due to the static capacity is not small enough to be neglected at the high frequencies there is only one alternative, that of lowering the specific resistance of the suspension, which may demand the use of other material in certain cases. From these considerations the cell shown in Fig. 2 was designed and found to fulfill all of the requirements.

A supply of saturated air at constant pressure was connected to tube A. The bubbles rising in B forced the suspension in that tube before them and gave rise to a circulation in a counter-clockwise direction, giving an entirely adequate stirring and at the same time allowing

a thorough oxygenation of the suspension. Considerable difficulty was experienced from persistence of bubbles; the surface tension of suspension was considerably different from that of sea water as was shown both by this persistence of bubbles at D and by increased size of the bubbles rising in tube B, Fig. 2. If these bubbles were not broken as soon as they appeared at the surface a froth was formed which contained a sufficiently large number of eggs to change the volume concentration noticeably. The only satisfactory method of breaking these bubbles was to place just above the surface of the suspension, at D, a "U"-shaped piece of glass rod which had previously been rubbed on the oily nose of the observer. The electrolytic cell proper C was connected by short pieces of rubber tubing at F F. The electrodes E E were of platinum covered with a very light and very fine deposit of platinum black plated on at as low a current density as possible. It was rather important that these electrodes be placed with their planes containing the axes of the side tubes, for otherwise there was a tendency for the eggs to pile up either above or below them. The cell used in this work has a constant of 5.85. No difficulty was experienced from either polarization capacity or static capacity, a perfectly constant impedance being obtained with sea water between the frequencies of 1000 cycles per second and 15 million cycles per second, D, Fig. 4. The pressure of the air supply was so regulated that there was always at least one bubble in the tube B. It was found that under these conditions about 10 cc. of air at atmospheric pressure passed through the cell per minute. The lowering of the impedance when there was no bubble in tube B was only 4 per cent, so it was assumed that the error introduced by the presence of the film of solution between the bubble and the glass wall of B was entirely negligible. The volume of suspension required was 4 cc.

Measuring Apparatus.—The measuring apparatus consisted of two Western Electric vacuum thermocouples connected to two Leeds and Northrup type R low resistance galvanometers. The deflections of both galvanometers were read by light spots thrown on the same scale.

In the vacuum thermocouples there is a small filament of metal or carbon known as the heater through which the current to be measured passes. Attached to the center of this heater element are two wires of dissimilar materials forming a thermocouple to measure the tem-

perature rise in the heater wire resulting from the heat produced by the current to be measured. This whole arrangement is placed in a glass bulb which is then evacuated. The evacuation greatly increases the sensitivity and at the same time makes the arrangement independent of small changes of room temperature. It is found that for small currents the E.M.F. of the thermo-junction is directly proportional to the square of the current flowing through the heater. For this work a couple having a 1000 ohm heater was used to measure the potential drop across the cell and the other thermocouple. This second thermocouple was used to measure the current through the cell and had a resistance of 34 ohms. It was soon found that this arrangement would not work at high frequencies, due to the fact that both galvanometers had a considerable capacity to ground while neither one was at ground potential. This state of affairs led to a more and more effective short circuiting of one-half of each of the heater elements as the frequency was increased beyond a certain point. The solution was to use only one-half of each heater element, Fig. 3—one of the thermocouple terminals serving for the other heater connection. In this way both galvanometers were at the same potential as the grounded side of the output from the oscillator. The high resistance thermocouple then served as an alternating current voltmeter and the low resistance thermocouple as an alternating current ammeter. When but one-half of a heater element is used the sensitivity of the device is multiplied by a factor of $\sqrt{2}$ when used as a voltmeter and divided by the same factor when used as an ammeter. From the constants of the thermocouples and the galvanometers it was found that

$$|Z| = 150 \sqrt{s_A/s_B} \text{ ohms.}$$

Experimentally it was found that

$$|Z| = 151 \sqrt{s_A/s_B} \text{ ohms,}$$

where s_A and s_B are the deflections of the voltmeter and ammeter galvanometers respectively, and $|Z|$ is the absolute value of the impedance.

The apparatus was checked over the entire range of frequencies by means of a specially designed shielded resistance whose impedance

was constant up to 10 million cycles, being in error by only 1.6 per cent at that frequency. It was found that the impedance of the electrolytic cell containing sea water, curve D, Fig. 4, was constant up to the highest frequency used, about 15 million cycles. The apparatus has also been checked by measuring the impedance of condensers and inductances.

In actual practice, the square root of the ratio of deflections of the two galvanometers was multiplied by a factor of 151 to give the impedance of the electrolytic cell plus the ammeter heater. This latter was a pure resistance of 18.4 ohms. At both the very high and the very low frequencies, the cell filled with a suspension of eggs had a zero phase angle and the resistance of the series heater could be subtracted directly to give the resistance of the cell suspension, but at the intermediate frequencies the phase angle was not zero and this fact had to be taken into account in the determination of the impedance of the cell suspension.

With the apparatus in its final form it was possible to take readings every 40 seconds over long periods and half of this time interval was consumed in recording the data. The actual impedance measurements had an accuracy of about 1 per cent. For full scale deflections the current through the cell was 0.002 ampere, and the potential drop across it 0.3 volt.

Data.

Of the living animal cells which were available, the eggs of the Echinoderm *Arbacia punctulata* seemed the most satisfactory because of their uniformly spherical shape, their hardness, and the possibility of using large numbers of them and having them keep "in step" for a considerable period after fertilization. The amount of investigation to which these eggs have been subjected and the availability of a moderate volume of eggs from a single specimen added to their desirability.

Over a thousand measurements of the impedances of suspensions of biological material were made at the Marine Biological Laboratory at Woods Hole during the summer of 1927. Although all of the analysis has been confined to the results on *Arbacia* eggs some data were taken on red blood corpuscles and on the eggs of *Asterias* and *Fundulus*.

Procedure.

The filament of the oscillator vacuum tube was usually turned on for at least 15 minutes in order to allow it to reach equilibrium before any readings were taken. Then a run was taken with the standard resistance to check the constancy of calibration of the thermocouples and galvanometers. A second run was taken with the electrolytic cell filled with sea water to determine the conductivity of the sea water and make certain that the error due to polarization capacity of the electrodes was negligible.

The ovaries of the *Arbacia* were removed entire from the specimen and placed in a finger bowl half filled with sea water. After standing for several minutes they were gently shaken with forceps and removed. The suspension was then carefully poured through wet unbleached muslin and allowed to settle. After settling the clear sea water was either pipetted or poured off, or else the eggs were brought to the center of the finger bowl by moving it in a circle and then pipetted into fresh sea water. In order to get rid of as much jelly as possible the suspension was gently poured from one finger bowl to another several times at each washing. Sometimes the eggs were centrifuged by hand just enough to throw them down to get the concentration desired before removing them to the electrolytic cell, but this procedure was avoided wherever possible. As soon as the suspension was placed in the cell the air line was connected and the stirring and aeration continued without interruption until the end of the experiment.

A set of readings usually consisted of the zero readings of both galvanometers with the oscillator disconnected and then the readings of both galvanometers at seven or eight different frequencies. After half a dozen or so sets of readings, the eggs were fertilized by the thorough stirring in of a small amount of "dry" sperm and readings continued until after first cleavage. In one or two runs the observations were continued up to 3 hours after fertilization but never longer than that. Very small samples were frequently removed from the cell for microscopic examination. At the end of the run the suspension was removed from the cell and centrifuged to determine the volume concentration of the eggs.

An entirely satisfactory technique for the handling of the eggs has

not been worked out as yet and while normal development was obtained in not over half of the runs made it was considered expedient to take some data on eggs—normal or abnormal—rather than to have normal development and no data. Polyspermy was probably the most common cause of abnormality and was not recognized as such

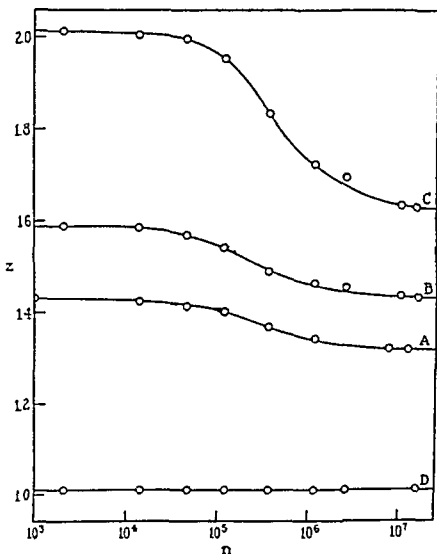


FIG. 4. Impedance (Z , arbitrary units) vs. log frequency (n , cycles per second) for sea water and *Arbacia* egg suspensions.

for some time due to the failure of the eggs to raise membranes although fertilized. The reason for this may be that in the concentrated suspensions used a slight cytolysis would elevate the osmotic pressure to such a degree that the membranes would not lift until the suspension was diluted with sea water.

Computations.

The general type of impedance variation with frequency is shown in the three curves of Fig. 4 for suspensions of *Arbacia* eggs in sea water. This change in impedance with frequency can mean but one thing, namely, some element or elements in the egg have an impedance which decreases as the frequency of the measuring current is increased. For the present it will be assumed that the *Arbacia* egg consists of a homogeneous electrolytically conducting interior having a very thin surface layer of vastly different electrical properties, which may be functions of frequency. Since the impedance approaches constant values at both the low and the high frequencies, R_0 and R_∞ , it is suggested that at the low frequencies the impedance of the surface is so high that it renders negligible the effect of the internal conductivity, whereas at high frequencies its impedance is so low as to be itself negligible. Having R_w , R_B , and ρ —the resistances of the sea water, B thermocouple heater, and the volume concentration of the eggs—as well as R_0 and R_∞ , it is possible to apply the Eq. (1) given in the previous paper¹ for the determination of an average specific resistance of the egg at both high and low frequencies,

$$\frac{r_1/r - 1}{r_1/r + 2} = \rho \frac{r_1/r_2 - 1}{r_1/r_2 + 2} \quad (1)$$

where r , r_1 , and r_2 are the specific resistances of the suspension, the suspending medium, and the suspended spheres respectively. For convenience the following notations have been employed,

$$\frac{\alpha_0 - 1}{\alpha_0 + 2} = \rho \frac{\beta_0 - 1}{\beta_0 + 2} \quad (2), \quad \frac{\alpha_\infty - 1}{\alpha_\infty + 2} = \rho \frac{\beta_\infty - 1}{\beta_\infty + 2} \quad (3),$$

where

$$\alpha_0 = \left(\frac{r_1}{r} \right)_0 = \frac{R_w - R_B}{R_0 - R_B}, \quad \beta_0 = \left(\frac{r_1}{r_2} \right)_0,$$

$$\alpha_\infty = \left(\frac{r_1}{r} \right)_\infty = \frac{R_w - R_B}{R_\infty - R_w}, \quad \beta_\infty = \left(\frac{r_1}{r_2} \right)_\infty.$$

¹ Cole, K. S., *J. Gen. Physiol.*, 1928, xii, 29.

For rapid calculation a curve of α vs. $\frac{\alpha-1}{\alpha+2}$ has been found very useful. From the α given by the data as shown above, the corresponding $\frac{\alpha-1}{\alpha+2}$ is found from this curve. When divided by the observed ρ , this gives $\frac{\beta-1}{\beta+2}$ from which β is found by using the same curve in the reverse direction. Since r_1 is known (usually about 25 ohm cm.) the absolute value of r_2 can be found.

By the above means β_0 and β_∞ have been found for all of the data taken and their values for the runs shown in Fig. 4 are given in Table I. The variations in β_0 and β_∞ are quite typical of all the data taken and are far greater than could be accounted for by errors in the electrical measurements. This conclusion is in accord with the observation

TABLE I.

	ρ	α_0	β_0	α_∞	β_∞	ρ'
	<i>per cent</i>					<i>per cent</i>
A	22.2	.733	.08	.804	.282	19.6
B	38.0	.676	.275	.763	.452	24.2
C	44.3	.477	.028	.647	.308	42.5

that the variations in both β_0 and β_∞ were always in the same direction from one run to another but that both were relatively constant during any one run. Eliminating ρ from Eq. (2) and Eq. (3) we have

$$\frac{\alpha_0 - 1}{\alpha_0 + 2} = K \frac{\alpha_\infty - 1}{\alpha_\infty + 2} \quad (4)$$

where K involves only β_0 and β_∞ and should thus be independent of ρ . A plot of $\frac{\alpha_0 - 1}{\alpha_0 + 2}$ against $\frac{\alpha_\infty - 1}{\alpha_\infty + 2}$ for all of the data gave a straight line passing through the origin with $K = 1.41$. This was the final check which verified the electrical measurements and placed all of the blame for the variations on the measurements of ρ , the volume concentration of eggs.

In Table I, ρ' is the volume concentration calculated from the

electrical data on the assumption of Eq. (6) of the previous paper¹ that $\beta_0 = 0$ or that at low frequencies the eggs are very poor conductors. The more reliable values of ρ indicate that this assumption is very close to the truth. For this case, from Eq. (4) we have

$$K = -\frac{1}{2} \frac{\beta_\infty + 2}{\beta_\infty - 1} \quad (5)$$

from which $\beta_\infty = r_1/r_2 = 0.28$. The specific resistance of the interior of the egg is then about 3.6² times that of the sea water or 90 ohm cm. As said before, ρ was obtained by centrifuging the suspension at the end of the run. This was never very satisfactory as it was desired to allow the eggs to develop further in order to determine their condition. Consequently the suspensions were centrifuged only enough to give what was thought to be "constant volume." The results show that this was not the case and several other methods of determining the volume concentration have been suggested and will be tried in future work. The formula has never been accurately checked with *Arbacia* simply because of this difficulty in the determination of ρ .

As a first approximation it has been assumed that the surface of the egg acts like a pure capacity, with $r_3 = 0$ —corresponding to Fricke's conclusion for the red blood corpuscle and Philipppson's assumptions for tissue. On this hypothesis, take Eq. (12) of the previous paper¹

$$\frac{x_3}{\gamma} = \sqrt{\frac{|z|^2 - r_\infty^2}{r_0^2 - |z|^2}} = s, \quad (6)$$

and s may now be computed directly from the impedance on suspensions of *Arbacia* eggs, as has been done in Table II and Fig. 5 for the runs plotted in Fig. 4. The straight lines are drawn arbitrarily with an intercept of 1.0 at $n = 3.10^5$ and a slope of -0.5 in each case. It will be noticed that the slope of each of these lines is about -0.5 and that s is not greatly affected by the volume concentration.

$|Z|$ has been computed from the two sets of data of Fricke and Morse

² Fricke and Morse (3) find the resistance of the interior of the red blood corpuscle to be 3.5 times that of the plasma.

(3) and the values of s are given in Table III and plotted in Fig. 6. The slope of the line is -0.93 .

Philippon's data (10) gives slopes of about -0.5 for animal tissues and -0.75 for vegetable tissues.

TABLE II.

A			B			C		
n	$ Z $	s	n	$ Z $	s	n	$ Z $	s
$1.0 \cdot 10^3$	1.433		$2.0 \cdot 10^3$	1.589		$2.0 \cdot 10^3$	2.015	
$1.35 \cdot 10^3$	1.425	3.84	$1.38 \cdot 10^3$	1.586		$1.38 \cdot 10^3$	2.008	
$4.45 \cdot 10^3$	1.414	2.3	$4.55 \cdot 10^3$	1.571	2.64	$4.55 \cdot 10^3$	1.99	5.1
$1.15 \cdot 10^4$	1.403	1.71	$1.16 \cdot 10^4$	1.542	1.56	$1.16 \cdot 10^4$	1.955	2.83
$3.48 \cdot 10^4$	1.368	.865	$3.59 \cdot 10^4$	1.491	.77	$3.59 \cdot 10^4$	1.834	1.08
$1.15 \cdot 10^5$	1.343	.51	$1.13 \cdot 10^5$	1.465	.51	$1.13 \cdot 10^5$	1.723	.56
$7.33 \cdot 10^5$	1.322		$2.5 \cdot 10^5$	1.454	.41	$2.5 \cdot 10^5$	1.695	.44
$1.2 \cdot 10^7$	1.317		$1.0 \cdot 10^7$	1.436		$1.0 \cdot 10^7$	1.634	
			$1.5 \cdot 10^7$	1.429		$1.5 \cdot 10^7$	1.626	

TABLE III.

n	$ Z $	s	$ Z $	s
$.087 \cdot 10^3$	191		332	
.833	180	2.03	317	2.04
1.17	172	1.43	306	1.435
1.52	166	1.155	297	1.135
2.04	157	.875	286	.88
3.04	146	.618	271	.613
3.82	140	.507	261	.456
4.52	136	.419	256	.371
	124		244	
$\rho = 46.0$ per cent $r_1 = 89.4$ ohm cm.			$\rho = 17.9$ per cent $r_1 = 291.0$ ohm cm.	

If π_3 were due to a perfect dielectric capacity c_3

$$\pi_3 = s \gamma = \frac{1}{c_3 \omega}$$

and the slope of the $\log s$ vs. $\log \omega$ curve would be -1.0 . This is in fair agreement with the data for red blood corpuscles but in no way

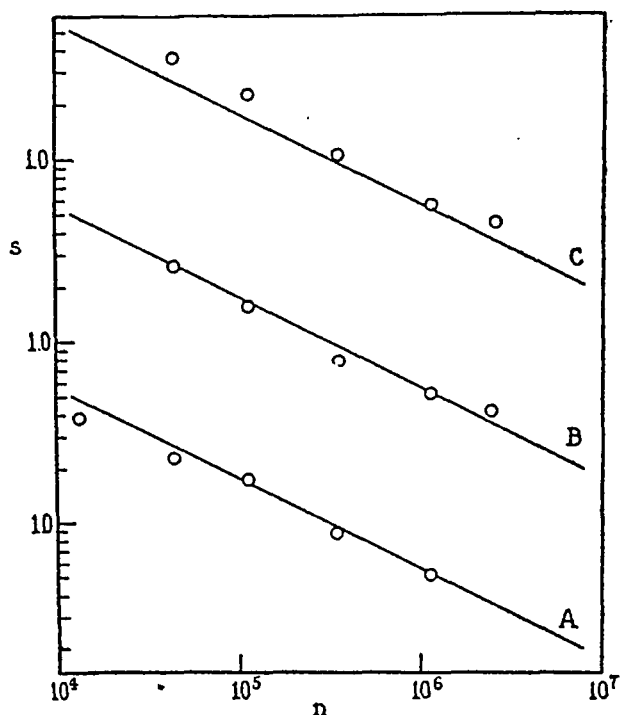


FIG. 5. $\log s$ (Eq. 6) vs. \log frequency (n , cycles per second) for *Arbacia* egg suspensions.

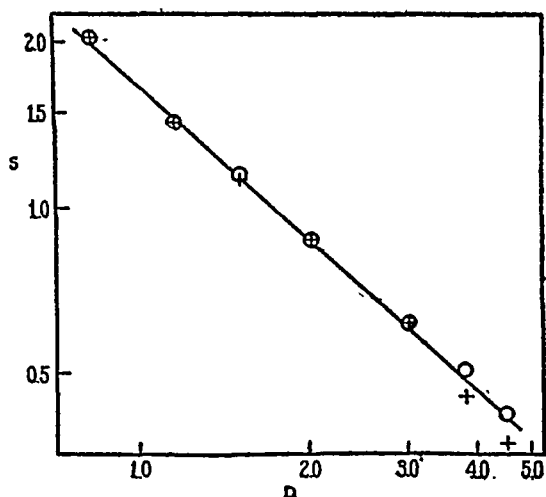


FIG. 6. $\log s$ (Eq. 6) vs. \log frequency (n , megacycles per second) for red blood corpuscle suspensions; data of Fricke and Morse.

agrees with that on tissues or on eggs—these giving capacities which vary inversely as the -0.25 and -0.5 power of the frequency. As stated in the previous paper, such variable capacities found in physical electrolytic systems have been classed as polarization capacities, and have associated with them an equivalent series polarization resistance. That the same is true for living systems is borne out by computations made on Gildemeister's data (4) for frog skin and on Blinks' unpublished data (1) for *Valonia*. For a single set of data for frog skin it was found that $m = 0.755$ and that the capacity varied as the -0.57 power of the frequency. For three sets of data it was found for *Valonia* that m was fairly constant for each case and varied from 0.63 to 0.75 . Also the capacities varied with frequency³ as a power having values from -0.13 to -0.23 . From the biological point of view we should expect $r_3 > 0$, since it may be expected to be more or less proportional to the permeability for ions—being an equivalent series resistance. In such cases, the initial assumption that $r_3 = 0$ is not valid, and it is necessary to go to the more general expression, Eq. (10),¹ for $|z_3|$. When impedance data alone are available it is impossible to use this relation without the assumption that m of Eq. (13) is constant. Even then, no convenient method of computation has as yet been found. It has however, been possible to show empirically by the calculation of hypothetical data that as m is allowed to take on different values in different cases—but assumed constant in each case—,

$$\frac{|z_3|}{\gamma} = s^\rho,$$

where ρ varies from one to two as m goes from zero to infinity. If

$$s = s_0 \omega^q$$

where $s_0 = s$ when $\omega = 1$, then

$$\frac{|z_3|}{\gamma} = s^\rho = s_0^\rho \omega^{\rho q}.$$

It follows therefore that if we assume $r_3 > 0$, and consequently $m > 0$, we find that $|z_3|$ instead of varying as the -0.5 or -0.75 power of ω , will vary as a power of ω more nearly -1.0 .

³ Cf. Osterhout (8).

Since there is good reason to believe that $m \neq 0$, and no certainty that it is constant, unsupported impedance measurements have no immediate decisive value. It may still be interesting, however, to calculate what this capacity might be if it were a capacity alone. Since the measurements of volume concentrations have been so very unsatisfactory it is convenient to use an expression for γ which does not involve ρ explicitly. It can be shown that

$$\gamma = \frac{r_0^2 (2 + r_1/r_0) (1 - r_1/r_0) a}{2 (r_0 - r_\infty)}.$$

This relation has been used in the calculation of x_3 in place of γ of Eq. (9) of the previous paper.¹ The values c_3 for n about $3.5 \cdot 10^5$ cycles per second are given in Table IV.

TABLE IV.

	r_0	r_∞	r_1	n	s	c_3
A	33.8	30.8	24.8	$3.48 \cdot 10^5$.865	$1.02 \mu f/cm.^2$
B	37.8	33.7	25.6	3.59	.77	1.03
C	52.3	38.8	25.0	3.59	1.08	.86

$$2a = 75 \cdot 10^{-4} \text{ cm.}$$

If we extrapolate to 10^3 cycles per second on the assumption that c_3 varies inversely as the 0.5 power of the frequency, we find $c_3 = 18 \mu f/cm.^2$

For red blood corpuscles Fricke found $0.8 \mu f/cm.^2$ which he computed would be the capacity of a monomolecular layer of oil if the static capacity formula used was assumed to hold for such dimensions. The value of $18 \mu f/cm.^2$ would thus lead to a layer only one-twentieth of this thickness at 1000 cycles and it would become "thinner" still at lower frequencies. This line of reasoning suggests that the barrier to ions at the surface of the cell—if a dielectric—must be of less than atomic dimensions, and leads one to the interesting possibility that the restriction on ionic transfer may be due largely to repulsive and attractive electrostatic forces of absorbed ions at the cell surface.

Fertilization.

In almost every run it was noticed that before insemination the values of the impedance for any given frequency were quite variable, giving rise to similar variations in the average specific resistance of the egg at both the high and the low frequencies. Immediately upon fertilization however, these quantities became quite constant and did not change noticeably thereafter. While it is possible that the temperature control was not adequate, it is more probable that the variations were due to some change in the average condition of the eggs in the suspension. It has been suggested that the cause may lie in the differing states of maturation of the eggs taken from different ovaries or different parts of the same ovary, and that the initiation of development completely masked these prefertilization irregularities. As a result it can only be said that there was found no specific change in the conductivity of the interior of the egg, or the impedance of the surface which can be definitely ascribed to the membrane formation. Previous work by Gray (6) and McClendon (7) has shown that there is a change in the low frequency conductivity of a centrifuged mass of eggs upon fertilization.⁴ It may very well be that the change is so small that it is beyond the limits of the present apparatus. It should, however, be pointed out that at low frequencies almost all of the current flows through the intercellular spaces since the surface of the egg offers a very high impedance to low frequency currents. Under these conditions a small change in the size of the eggs (5) or in the conductivity of the intercellular liquid would cause a considerable change in the conductivity of the centrifuged mass, as has been pointed out by others. At low frequencies, $|z_3|$ may be so large that—even when it varies considerably—Eq. (6) of the previous paper¹ should be true. While this equation cannot be expected to hold except for small values of ρ , it is worth noticing that if ρ is near unity, as it would be for a centrifuged mass of eggs, r_0 varies rapidly with both ρ and r_1 .

⁴ In conversation, Dr. Hugo Fricke said that he had found a change in the capacity of a frog egg upon fertilization but gave no further details. Dr. Sterne Morse stated, also in a conversation, that he had found a change in the capacity of a suspension of *Arbacia* eggs upon fertilization that he thought could be measured. He was working with a capacity bridge at 1000 cycles.

SUMMARY.

Apparatus has been designed and constructed for the measurement of the electric impedance of suspensions of *Arbacia* eggs in sea water to alternating currents of frequencies from one thousand to fifteen million cycles per second. This apparatus is simple, rugged, compact, accurate, and rapid.

The data lead to the conclusions that the specific resistance of the interior of the egg is about 90 ohm cm. or 3.6 times that of sea water, and that the impedance of the surface of the egg is probably similar to that of a "polarization capacity". The characteristics of this surface impedance can best be determined by measurements of the capacity and resistance of suspensions of eggs.

No specific change has been found in the interior resistance or the surface impedance which can be related either to membrane formation or to cell division.

The interest and assistance of Professor E. L. Chaffee and Professor W. J. Crozier have been very much appreciated. The author is indebted to Mr. P. S. Bauer for the use of his wavemeter and microscope.

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STUDIES ON PERMEABILITY OF MEMBRANES.

V. THE DIFFUSION OF NON-ELECTROLYTES THROUGH THE DRIED COLLODION MEMBRANE.

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Several investigators have already studied the diffusion of non-electrolytes across membranes with very narrow types of pores (1-3). As their experiments were carried on with Traube's copper ferrocyanide membrane or with bag shaped dried collodion membranes there were certain mechanical difficulties due to delicacy of the membranes themselves which prevented any great extension of the investigations. In general, however, the experiments did tend to establish the fact that the diffusion rate of a substance was much more strongly dependent on molecular weight or more accurately molecular volume when a membrane was present than when one was not.

In the present investigation we have been able to use the exceedingly durable flat type of dried collodion membrane described in one of our earlier papers (4). We have restricted ourselves to a small number of substances, attempting to study each under the best conditions available. Acetone, urea, glycerine, and glucose were selected as best suited to our purposes because these substances can be obtained in pure form and because quantitative estimation by microanalytical procedures is possible.

Arrangement of Diffusion Experiments.

In all of the experiments the arrangement of apparatus was essentially the same. A moist chamber large enough to hold four or five membranes was used to contain the outside solution. The membranes placed in this were kept from resting on the bottom by a frame of glass rods. At the beginning of an experiment about 20 cc. of distilled water was placed inside of each glass bell supporting a membrane, a solution of the substance to be investigated in desired concentration

poured into the outside chamber, and the membranes immersed in the solution. Evaporation was prevented by a tightly fitting lid. After a suitable time the liquid inside the membranes was removed and the amount of substance diffused from the solution on the outside determined. A fresh quantity of water was immediately placed inside the membranes which were returned to the chamber at once, so that a new experiment was begun at the conclusion of each preceding one. Thus, when a stationary rate of diffusion had been reached, that is, when the diffusing substance had established a stationary gradient across the membrane, as little as possible was done to disturb the gradient, and a series of experiments conducted to determine as accurately as possible the rate of diffusion.

Due to causes which will be discussed later the establishment of a stationary diffusion rate was often exceedingly slow. After we had recognized this fact it became customary to wait several weeks, during which time the liquids were frequently changed, before beginning the analyses. A relatively close agreement between the results of successive experiments was taken as an index of the establishment of the stationary condition.

In working with urea and glucose it was possible to prepare an outside solution of the exact concentration desired. With acetone and glycerine, however, it proved more convenient to make up the solutions in approximately the concentration desired and then determine their exact strength by analysis during the course of the experiments. Especially was this necessary in the case of acetone. Acetone is extremely volatile and evaporates from solutions so rapidly that it was impractical to maintain a solution of any definite concentration. When working with acetone special care was taken to see that the lid of the chamber containing the solution fitted tightly. The exact strength of the solution was determined by analysis at the beginning and end of each experiment and the average figure taken as the concentration during the experiment. With other substances the depth of the solution in the chamber was from 2 to 3 cm. With acetone a depth of about 15 cm. was used so as to lessen the relative surface exposed for evaporation. Moreover the bells supporting the membranes were tightly stoppered so that no acetone might reach the inside solutions by way of vapor in the chamber. Even with these precautions a steady decrease in the concentration of the acetone solution could be observed but it was very slight during the course of any one experiment. From time to time an amount of acetone equivalent to that lost by evaporation was readded to the solution.

The first substances with which we worked were acetone and glycerine. These early experiments were conducted at room temperature, the slight effect of daily variations being neglected. Later an ice box, maintaining and recording a constant temperature of about 2°C., became available and was utilized for all the experiments with glucose and urea and for many further experiments with acetone and glycerine. Besides providing a constant temperature the coldness of the ice box was of material assistance in preventing decomposition of the organic substances by bacteria.

Methods of Analysis.

Acetone.—Acetone was determined iodimetrically in most cases with 0.01 *N* solutions of iodine and sodium thiosulfate. When titrating with solutions of such low titer it is especially important to remember that in alkaline solution the hypiodite ion passes over easily to the iodate ion by dissolved oxygen. It was therefore necessary to make parallel blank determinations under accurately corresponding conditions and to correct for this blank. In some of the experiments the acetone had to be determined in the presence of glycerine. As even the purest glycerine we were able to obtain contained traces of substances combining with iodine, the acetone was transferred from the glycerine containing solution into the alkaline hypiodite solution by the aeration method of Folin (5). The parallel blank consisted in replacing the glycerine-acetone solution by one of glycerine only. Fortunately the iodine-combining substances of the glycerine were non-volatile.

Urea.—The urea used showed a negative test for ammonia with Nessler's reagent. Exposed to the air, even at the temperature of the ice box, such a solution gave a positive reaction after several days but it could be shown that this was due not to decomposition of urea but to ammonia in the air. In hermetically tight bottles no Nessler reaction occurred after several weeks. In performing our experiments the outside solution was frequently changed. Moreover, in preparation for analysis the inside solution was evaporated almost to dryness on the steam bath at neutral reaction, a process sufficient to remove traces of ammonia. The nitrogen was then determined by the micro Kjeldahl method (Direct Nesslerization method of Koch and McMeekin (5)). Control analyses with known amounts of urea under the same conditions showed the efficiency of the method.

Glycerine.—Glycerine was determined by the reduction of a standard chromic acid solution. The method used was derived from the procedure described by Bang (6) for the determination of fat in blood. The chromic acid solution was carefully standardized against solutions of pure glycerine made to known strength by specific gravity readings. We found 1 cc. of 0.1 *N* chromic acid solution equivalent to 0.00713 millimols of glycerine. This factor is in substantial agreement with that given in an analogous method used in the arts for the determination of crude glycerine (7). Our procedure was as follows: The solution of approximately 25 cc. volume was evaporated on a steam bath. We were able to show that evaporation caused no loss of glycerine provided it was not continued beyond a volume of 0.5 to 1 cc. Complete evaporation of the water resulted in losses of 10 per cent or more of the total glycerine. A sufficient quantity of 0.1 *N* chromic acid (usually not more than 2.0 cc.) was run in from a burette and 5 cc. of concentrated sulfuric acid added for each 2 cc. of chromic acid taken. When insufficient chromic acid had been used as shown by the change in color more was added and correspondingly more sulfuric acid. Complete oxidation of glycerine occurs within several minutes but usually we waited an hour before washing the

TABLE I.

Date	Membrane											
	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	F-9	F-10	F-11	I-1
	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.	mv.
June, 1927	42.2	28.1	45.4	38.3	36.0	15.4	29.7	47.0	41.1	38.5	32.1	47.0
July, 1927	41.8	26.3	45.4	38.2	33.7	16.3						47.1
Sept., 1927	39.2	24.2	40.4	32.6	28.2	8.9	30.1	46.3	41.5	39.0	35.5	48.3
Dec., 1927	35.7	22.2	35.6	28.1	23.2	12.3	25.8	40.3	36.1	34.2	25.7	47.1
Feb., 1928					12.5							45.4
												49.6
												48.0

Shows potential differences between 0.1 N and 0.01 N KCl solutions (*Co P*) with the membranes used in this investigation at various times during the period of study. These *Co P* readings have been taken as an index of the degree to which the initial properties of the membranes were maintained.

solution into a large beaker, adding several cubic centimeters of 5 per cent potassium iodide solution, and titrating the liberated iodine with 0.1 N sodium thiosulfate solution.

Glucose.—Glucose was determined by the method of Hagedorn and Jensen (5) used for blood, omitting the steps concerned with the removal of protein.

Constancy of the Membranes.

The reproducibility of the potential difference given by a membrane between 0.1 N and 0.01 N potassium chloride solutions (*Co P*) was taken as a gauge of the constancy of the properties of the membrane. In Table I we have arranged the *Co P* values of the membranes used in this study at the beginning of the experiments and subsequent readings made from time to time during the period. The method of making these readings has been previously described. In general it required a week before a constancy of potential within a millivolt was reached. After this time several readings were made on successive days and the average figure entered as the correct *Co P* value. It will be seen that in most of the membranes a drop of several millivolts occurred during a period of 6 months while in a few the *Co P* value remained essentially unchanged. It may be that some of the minor discrepancies to be reported later may be attributed to these slight changes in the membrane properties.

Among the substances with which we worked there is one, acetone, which in high concentration can act as a solvent for collodion. One can suppose that in more dilute solutions a trace of this action might remain, that the acetone might cause the collodion to swell and alter somewhat the properties of the membrane. In other words it might be suspected that in this particular case the membrane might function not only as a sieve but also as a solvent and so alter the mechanism of diffusion. In the concentrations actually used in our experiments, in only a few cases greater than 0.1 N or about 0.6 per cent, the property of acetone as a solvent for collodion could neither be expected nor observed. However, in order to establish the insignificance of acetone in these low concentrations on the membrane properties *Co P* measurements were made with one membrane in which the two potassium chloride solutions on the two sides of the membrane contained acetone in 0.1 M concentration. The ordinarily obtained value

for the $Co P$ of this membrane was 50 millivolts. In the presence of acetone it was 48 millivolts. The original value was reobtained when the acetone-containing potassium chloride solutions were replaced by pure potassium chloride solutions. The change was very slight and reversible. Without attempting a definite explanation for the slight change observed it may be mentioned that the effect of acetone on the activity coefficient of potassium chloride at different concentrations is quite sufficient to account for the small difference.

Selection of Form for Expressing Results.

If the ordinary rules of diffusion are applicable the amount of a substance which will diffuse through a membrane is proportional to the time, to the exposed area, to the difference in concentration existing between the solutions at the two borders of the membrane, and inversely to the thickness of the membrane or length of the pore channels. The constant which converts this proportionality into an equation is called the diffusion coefficient. In these investigations we are to deal with a comparison of the rates at which two different substances diffuse through the same membrane and in making such a comparison may regard the exposed area and membrane thickness as constant. Moreover, the time of diffusion was never long enough to alter appreciably the difference in concentration between the liquids on the two sides of the membrane and this difference may likewise be considered a constant. For this reason it will be sufficient for our purposes to express the rate of diffusion for each membrane with each substance in a definite concentration in millimols passing through the membrane per hour. Inasmuch as we are presently to demonstrate that the rate of diffusion with our membranes is proportional to the difference in concentration, that is, that this principle of Fick's law holds also for the dried collodion membrane, it will be possible to express all diffusion rates in terms of the rate that would hold for some fixed reference concentration. For convenience throughout this paper we shall express our results in terms of the diffusion rate holding for 0.1 M solutions.

Concerning the effect of temperature it may be stated that the factor of dependence is not large and that in view of other experi-

mental errors incident to these experiments a variation of 1° or 2°C . is of negligible significance. In the experiments conducted in the ice box a constant temperature was maintained.

Dependence of Diffusion Rate on Difference in Concentration between Solutions on Two Sides of Membrane.

Our first problem was to show that the rate of diffusion for any membrane was proportional to the difference in concentration between the two solutions on the two sides of the membrane. We did not feel that we would be justified in speaking of a diffusion coefficient in the true sense of Fick's law unless such a relation could be shown to exist. Furthermore it was evident that much economy of time would result if we were able to conduct experiments with the more slowly diffusing substances in higher concentrations and if all results could then be referred to a common unit.

The first suggestive proof that such a definite relation between concentration and rate of diffusion did exist was furnished by a few experiments with glycerine in 0.5 M and 0.1 M concentrations. Four different membranes were used. The result is shown in Table II. The average diffusion rate for the four membranes was 2.00×10^{-5} millimols per hour for the experiments in 0.5 M concentration when expressed in terms of a supposedly proportional rate for 0.1 M concentration and 2.20×10^{-5} millimols per hour for the experiments actually conducted at this concentration. The agreement is as good as can be expected when one considers the small amounts obtainable for analysis at the lower concentration.

In another series of experiments with acetone done at the same time the diffusion coefficients obtained from the experiments in 0.5 M concentration and those in 0.1 M concentration were essentially the same but the experiments in more dilute solutions led to higher relative rates. We surmised that this discrepancy was the result of experimental errors rather than real and accordingly planned another series of experiments with acetone in which the region of concentration which had previously shown the discrepancy was specially investigated. In this series care was taken to eliminate the supposed sources of error in the first series. A period of 3 weeks was allowed for the establishment of the stationary diffusion gradient in the more dilute of the

solutions before any analyses were made. A constant temperature was maintained in the ice box. The results of this series are shown in Table III. Although the rates as determined from individual experiments, especially those in 0.01 M concentration, vary considerably, a comparison of the average rates from each group of experiments shows a striking uniformity. The average of the four membranes shows a rate of 28.9×10^{-5} millimols per hour determined when the concentration of the outside solution was 0.1 M and a relative rate of 29.6×10^{-5} millimols per hour calculated from the experiments in 0.01 M concentration.

It is necessary at this point to call attention to the rather large discrepancies occurring between the results of individual experiments. These discrepancies are far beyond the limits of error of the analytical

TABLE II.

Membrane	F-3	F-5	F-6	C-7
Diffusion rate observed in 0.5 N concentration.	8.04	3.94	15.45	12.45
0.1 N diffusion rate calculated from above...	1.61	0.79	3.09	2.49
Observed 0.1 N diffusion rate.....	1.56	0.77	3.56	3.02

Experiments with glycerine indicating that the diffusion rate is proportional to the difference in concentration between the inside and outside solutions. All rates are expressed in terms of millimols $\times 10^{-5}$ per hour.

methods employed. They were observed throughout all the experiments of this study,—with urea, glycerine, and glucose as well as with acetone. Moreover, it will be observed that with some membranes the tendency to show variations in this respect is much greater than in others in which the results of individual experiments are fairly uniform. In the experiments in 0.01 M concentration listed in Table III the results with the membrane F-7 show an average deviation from the mean rate of 30.9 per cent, while in the experiments with the membrane F-9 the average deviation is only 5.4 per cent. These considerations have led us to believe that the tendency to discrepancy between the results of individual diffusion experiments is an inherent property of the membrane itself. An explanation of this phenomenon will be attempted later. For the present it is sufficient to call attention to its existence as an explanation for the adopted method of ob-

TABLE III.

Membrane	Rate calculated from experiments in 0.01 M concentration	Average rate from 0.01 M experiments	Rate calculated from experiments in 0.1 M concentration	Average rate from 0.1 M experiments
F-7	19.9	39.5	35.1	34.1
	21.7		31.8	
	51.0		35.4	
	46.6			
	34.3			
	54.2			
	48.8			
F-8	25.8	21.4	22.0	21.9
	20.6		22.9	
	21.9		20.8	
	15.2			
	22.3			
	20.2			
	23.9			
F-9	37.9	35.5	37.4	38.2
	34.0		40.8	
	39.9		37.0	
	32.3		37.5	
	33.7			
	35.4			
	35.6			
F-10	33.5	21.8	20.3	21.4
	18.9		24.2	
	22.7		21.2	
	17.4		20.0	
	20.9			
	17.4			

Second series of experiments with acetone to show the relation between difference in concentration on the two sides of the membrane and rate of diffusion. Results of individual experiments are given together with average results for each series of experiments. All experiments performed at constant temperature of ice box. All rates are expressed in terms of a supposedly proportional 0.1 M rate and as millimols $\times 10^{-5}$ per hour.

TABLE IV.

Membrane	Rate of diffusion of acetone	Average acetone rate	Rate of diffusion of glycerine	Average glycerine rate	Ratio of rates acetone rate glycerine rate
F-1	104.0	123.0		1.71	71.9
	122.8				
	133.1		1.95		
	116.7		1.80		
	124.7		1.44		
	130.5		1.66		
	117.6				
	134.2				
F-2	80.2	83.2		1.50	55.5
	78.7				
	83.6		1.60		
	100.2		1.58		
	69.0		1.53		
	102.1		1.28		
	68.1		1.50		
	83.9				
F-3	140.2	125.3		1.56	80.3
	104.4				
	113.9				
	144.5		1.82		
	141.6		1.40		
	156.0		1.45		
	101.5				
	109.1				
	116.7				
	136.1				
	114.8				
F-4	75.9	92.1		1.66	55.5
	97.7		1.84		
	89.1		1.75		
	99.5		1.60		
	73.4		1.60		
	102.9		1.49		
	94.6				
	104.0				

Shows two series of experiments in which rate of diffusion was determined with both acetone and glycerine. Last column indicates the ratio of these rates. Majority of acetone experiments were conducted in a concentration of approximately 0.1 M; majority of glycerine experiments in a concentration of approximately 0.5 M. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour. Experiments conducted at room temperature.

TABLE IV—*Concluded.*

Membrane	Rate of diffusion of acetone	Average acetone rate	Rate of diffusion of glycerine	Average glycerine rate	Ratio of rates $\frac{\text{acetone rate}}{\text{glycerine rate}}$
F-5	78.8	76.8	0.68 0.89 0.73 0.80	0.78	98.5
	63.9				
	80.2				
	81.8				
	86.7				
	81.3				
	60.9				
	56.7				
	95.0				
	82.6				
F-6	79.6	77.6	2.92 3.26 4.69 2.43	3.33	23.3
	58.2				
	71.1				
	70.3				
	79.6				
	75.3				
	76.7				
	79.4				
	105.0				
	80.8				
C-6	181.8	167.4	7.47 7.21 7.85 6.92 6.99 5.77	7.04	23.8
	190.6				
	134.9				
	160.7				
	120.9				
	120.7				
	188.9				
	163.8				
	160.1				
	221.7				
C-7	203.6	243.5	2.49 2.11 3.92	2.84	85.7
	161.6				
	237.0				
	251.3				
	318.5				
	305.7				
	314.0				
	258.2				
	183.1				
	213.4				
	187.9				
	216.9				
	193.0				

taining the diffusion rate in any particular case from the mean result of a series of experiments rather than from a single experiment.

TABLE V.

Membrane	Average diffusion rate of acetone	Rate of diffusion of urea	Average urea rate	Ratio of rates $\frac{\text{acetone rate}}{\text{urea rate}}$
F-7	36.8	16.3	13.6	2.71
		12.5		
		13.7		
		14.8		
		10.8		
F-8	21.7	17.5	17.2	1.26
		14.6		
		16.3		
		24.1		
		13.7		
F-9	36.9	29.4	28.0	1.32
		25.8		
		27.0		
		32.1		
		25.7		
F-10	21.6	18.1	16.8	1.29
		13.8		
		18.0		
		21.9		
		12.4		

Shows two series of experiments in which the rate of diffusion was determined with both acetone and urea. With urea the results of individual experiments and the average result are given. With acetone only the average result is given; the results of the individual experiments were given in Table III. Urea experiments were conducted in a concentration of 0.1 M. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour. Experiments conducted at constant temperature of ice box.

Experiments to Determine Relative Rates at Which Various Non-Electrolytes Diffuse through the Dried Collodion Membrane.

In these experiments we did not follow the method used by Fujita in which mixtures of two different substances were placed in solution

on one side of the membrane and distilled water on the other side and the relative quantities of the two substances diffusing through the membrane determined. Because of the durability of our membranes we were able to work with one substance at a time, measuring as accurately as possible by a series of experiments the diffusion rate for each substance. We then compared the rates at which the different

TABLE VI.

Membrane	Rate of diffusion of urea	Average urea rate	Rate of diffusion of glycerine	Average glycerine rate	Ratio of rates urea rate glycerine rate
C-1	644.1	597.6	26.2	28.0	21.3
	552.4		31.2		
	596.4		29.3		
			25.4		
C-2	570.8	546.4	84.9	92.4	5.9
	511.8		100.6		
	556.5		91.8		
F-11	16.7	13.8	0.54	0.52	26.5
	10.4		0.52		
	14.3		0.51		

Shows two series of experiments in which the rate of diffusion was determined with both urea and glycerine. Last column indicates the ratio of these rates. Urea experiments were conducted in a concentration of 0.1 M; glycerine experiments in a concentration of approximately 0.5 M. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour. Experiments conducted at constant temperature of ice box.

substances diffused. By this method we eliminated the possibility of changes in the diffusion rates due to the mutual action of two substances on each other.

Acetone-Glycerine.—The results of experiments in which the diffusion rates for acetone and glycerine were compared have been listed in Table IV. It will be seen that with the membranes used the ratio of the diffusion rates varied between 23 to 1 and 98 to 1, acetone being the more rapid. This difference is far beyond any sources of error. Even with the membrane giving the smallest figure the ratio is many times larger than would be expected if no membrane were present.

With no membrane, that is in free aqueous diffusion, the rates vary approximately inversely with the square root of the molecular weight. In this case the ratio of the rates would be 1.26 to 1.

Acetone-Urea.—In Table V have been listed the results of experiments comparing the diffusion rates of acetone and urea. It will be seen that acetone diffuses 1.3 to 2.7 times as fast as urea. The molecular weight of urea (60) is slightly greater than that of acetone (58). We cannot insist that this slight difference is sufficient to account for the difference in the diffusion rates for an accurate comparison would

TABLE VII.

	Membrane					
	C-1	C-6	C-7	F-2	F-8	F-11
Diffusion rates from individual experiments	0.83	1.28	0.17	0.49	0.58	0.37
	0.61	2.00	0.22	0.56	0.66	0.43
	0.42	1.36	0.28	0.60	0.52	0.36
	0.46	1.22	0.17	0.89	0.49	0.29
	0.55	1.65	0.20	0.65	0.43	0.33
	0.44	1.70	0.22	0.67	0.38	0.35
	0.51	1.79	0.22	0.83	0.67	
	0.87				0.70	
	0.85				0.77	
					0.33	
					0.17	
					0.24	
					0.25	
Average rate.....	0.616	1.57	0.211	0.670	0.476	0.355

Results of diffusion experiments with glucose. Performed at ice box temperature and with outside solution of 0.5 M concentration. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour.

have to depend on a knowledge of the molecular volume, water envelope and intermolecular forces, and these only approximately depend on the molecular weight. We shall merely state that these two substances with almost the same molecular weights have also approximately the same diffusion rates.

Urea-Glycerine.—In addition to the experiments with acetone and glycerine and those with acetone and urea which in themselves permit us to form an estimate of the urea-glycerine diffusion ratio, a few

experiments were performed in which the diffusion rates of these two substances were directly compared. These have been listed in Table VI. It will be seen that urea diffused 6 to 26 times as rapidly as glycerine.

Glucose.—No separate set of experiments was done in which the diffusion rate of glucose was compared with one other substance.

TABLE VIII.

Membrane	Average acetone rate in pure solution	Rate of diffusion of acetone in presence of glycerine	Average acetone rate in presence of glycerine
F-3	125.3	120.3 127.6 143.1	130.3
F-5	76.8	66.1 82.1 90.2	79.5
F-6	77.6	63.0 59.9 72.7	65.2
C-7	243.5	231.4 280.7 206.6 330.7	262.4

Shows results of two series of experiments in which the rate of diffusion of acetone was determined. In the first series (given in detail in Table (IV)) pure water was placed inside the membranes and approximately 0.1 M acetone solution outside. In the second series the concentration of the outside solution remained 0.1 M with respect to acetone but in addition both inside and outside solutions were 0.1 M with respect to glycerine. All rates reduced to terms of 0.1 M and expressed as millimols $\times 10^{-5}$ per hour. Experiments conducted at room temperature.

Rather we selected membranes used in previous experiments with which the diffusion rates of two other substances were already known. The results of the individual experiments are listed in Table VII. By comparing the diffusion rates of glucose with the rates for other substances given in the previous tables, it will be seen that the rate with glucose was always much smaller than with any of the other sub-

stances studied. Later we will list and discuss the ratios of the glucose rates to each of the other substances.

Experiments to Determine the Effect of One Substance on the Diffusion Rate of Another.

Inasmuch as in his experiments Fujita (2) had worked with mixtures of two substances, we thought it worth while to find out whether the presence of one substance would change the diffusion rate of another. It was conceivable that some of the membrane pores might become clogged with the molecules of one substance and that fewer pores would be available for the diffusion of the other. For this purpose we determined to see whether the rate of diffusion of acetone could be altered by the presence of glycerine. Four membranes were selected in which the rate of diffusion of acetone was accurately known. These membranes were allowed to stand for several weeks in contact on both sides with a 0.1 M solution of glycerine. Then enough acetone was added to the outside solution to make it 0.1 M with respect to acetone as well as glycerine, and after a suitable time allowed for the establishment of a definite diffusion gradient, the rate at which the acetone diffused through the membranes in the presence of glycerine was measured. The results have been arranged in Table VIII. It will be observed that we were unable to detect any effect of the glycerine on the rate of diffusion of acetone.

Experiments to Indicate Steps Involved in Establishment of Stationary Diffusion Gradient.

It has already been pointed out that in these diffusion experiments we found it necessary to wait some days for the establishment of a definite diffusion gradient before any consistency of results could be obtained. It seemed highly desirable that we should have some idea of the processes going on during the establishment of this definite gradient. With the substances with which we first worked only rough ideas could be obtained as the analytical methods available were not sufficiently sensitive to determine quantitatively the small amounts diffusing in the first few hours. With acetone we did observe that in general the result obtained from a first or second experiment

was lower than later ones. However, when we began to work with the extremely accurate micro methods available for the determination of sugar it became possible to make such studies quantitatively even though the diffusion coefficient of sugar is many times smaller than that of acetone. Experiments were begun with membranes which had been kept in water for several weeks previously and which had never been in contact with sugar. A 0.5 M glucose solution was used. From the first we determined at regular intervals the quantity of sugar passing the membrane. From these figures it was possible to

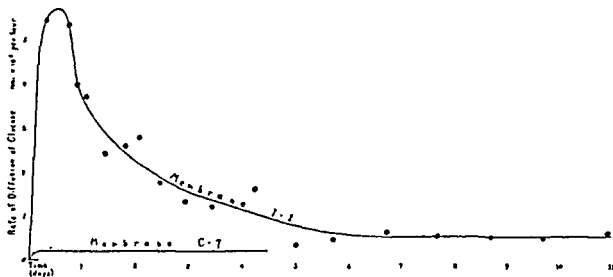


CHART 1. Shows the various diffusion rates holding during the period of establishment of the stationary diffusion gradient of glucose with two different membranes. The dots represent the results of individual experiments, each dot being charted in the middle of the time period over which the diffusion was measured. The curves have been drawn by interpolation.

construct a curve showing the changes in diffusion rate during the establishment of the stationary gradient. In Chart 1 we have drawn two curves illustrative of two different types of processes involved. In the experiments with the membrane I-1 the most rapid rate was obtained from the first experiment. After this the rate decreased from day to day reaching a stationary value at about the 6th day. We have drawn the first part of the curve beginning at zero because it is evident that some time must have elapsed before any glucose could have crossed the membrane. The impression gathered from this curve is that the process is similar to the one observed when a protein

solution is filtered through an ultrafilter,—the pores of the filter gradually becoming clogged. Here it would seem as though the glucose molecules had gradually clogged or plugged up many of the channels in the membrane which were available at first. In contrast to this curve stands the one obtained with the membrane C-7. Here the diffusion rate was quite constant from the beginning.

DISCUSSION.

In offering an explanation for the large differences in the diffusion rates of different substances through the same membrane there are two theories requiring consideration. On the one hand we may conceive of the membrane as a phase working as a solvent for the diffusing substance. In this case the partition coefficient would determine the gradient of concentration of the substance in the membrane and thus control the rate of diffusion. On the other hand it is possible that the specific mobility of the substance expressed as a diffusion coefficient has a different value within the membrane from that in water. In this case it is not necessary to employ a concept of solubility although the two theories do not exclude each other. At first sight the large differences between the diffusion rates with different substances would seem to favor the hypothesis that the rate is determined in the greater part by solubility. This is because we are accustomed to large differences in solubility between different substances but not to large differences in mobility. For this reason Northrop (8) recently gave preference to the solubility theory. In previous papers we have treated the dried collodion membrane as a sieve membrane with pores almost of molecular size. When the membrane channels are as minute as this a distinction between a molecule in solution in the membrane and one within a channel becomes doubtful. A full discussion of this problem of greatest importance to an understanding of membrane action would lead us far from the purpose of this paper. In a later communication we shall bring together a number of facts which enable us to give preference to the theory of sieve action and during the present paper will accept this theory without further discussion.

In attempting then to form a mental picture of the mechanism of diffusion in our experiments which would account for the observations

made two possibilities were kept in mind. We thought it probable that the great difference in the diffusion rates between molecules of different sizes was due to the fact that the membranes contained pore channels of varying sizes, that only the largest of these channels would serve for the passage of a large molecule such as glucose, and that many more channels could be utilized by the smaller molecules such as acetone and urea. Now it seems rational to suppose that the quantity of any of these substances which can diffuse in a given time will be proportional to the total area of all the pores large enough to function in its transport. This area may be called the available pore area (A.P.A.) for a substance in contradistinction to the total pore area of the membrane. The A.P.A. evidently must become larger as the molecular size decreases. An example may be more easily followed than a general discussion. In the case of glycerine and acetone the data in Table IV will show that on an average acetone diffused 62 times more rapidly than glycerine. In free aqueous solution without a membrane acetone would diffuse only 1.26 times as rapidly as glycerine. We may then state that the effect of the membrane was to decrease the rate of glycerine diffusion 49 times more than it decreased the acetone rate ($49 \times 1.26 = 62$). That is, the A.P.A. for acetone must be 49 times as great as the A.P.A. for glycerine or because the former includes the latter as one of its parts it follows that only 2 per cent of the A.P.A. for acetone is available for glycerine. Similarly it can be shown by analysis of the figures in which glycerine and glucose were compared that only 10 per cent of the A.P.A. for glycerine was available for glucose and from the experiments with acetone and glucose that only 0.3 per cent of the A.P.A. for acetone could be utilized by glucose. Such differences as these can exist only if the number of pores utilizable by the substances of smaller molecular size are many times greater than the number of larger pores and we may conclude that in general as the size of the pore channels increases the number of these channels greatly decreases.

However it is possible to formulate another hypothesis in which the great difference with which molecules of larger and smaller size diffuse may be attributed to increased frictional resistance of the larger molecules against the pore walls rather than to variations of pore size. The experiments just described have led us to believe that this explana-

tion is not the major one. We have seen that a membrane saturated with glycerine has essentially the same permeability to acetone as in the absence of glycerine. If the same membrane channels were being utilized for the passage of glycerine as for acetone it is hard to believe that the more slowly moving glycerine molecules would not greatly interfere with the diffusion of acetone. On the other hand, if the first hypothesis is the correct one, the smaller pores cannot be utilized by glycerine and their channels will remain open even in the presence of a high concentration of this substance and can function for the transport of acetone. As the pore area that can be entered and clogged by the glycerine molecule is only 2 per cent of the A.P.A. for acetone it follows that 98 per cent should still be able to function in carrying acetone. Because of this it is scarcely to be expected that we would be able to detect any effect on the rate of diffusion of acetone and this is in accordance with the observations.

Again we have called attention to the fact that with some membranes during the period of establishment of the stationary diffusion gradient the rate at which glucose diffuses is more rapid during the first days or hours than later when the rate has become constant. It is not possible to account for this observation on the assumption that friction alone is responsible for the slow rate of diffusion of glycerine. We have explained it upon the basis of the first hypothesis by supposing that some of the membrane channels are of such a size that they are permeable to glucose for a short time only. Gradually as the diffusion continues they become clogged and the final constant diffusion rate is maintained by pores of slightly larger size which do not become clogged. We are conscious of the fact that the statement that a membrane channel has been clogged with a molecule may not mean that the movement of this molecule has been stopped entirely but merely that its progress has been hindered for a time and that such a conception would be equivalent to admitting that friction does play a part. However, we could not on this basis, account for the regular way in which the diffusion rate decreased from day to day until a stationary rate was reached. It is possible, and we believe probable, that the final stationary rate represents a condition not only in which some pores have remained permanently open and some permanently closed but also in which another group of pores are being

temporarily occluded and then reopened in such a manner that more or less of an equilibrium is maintained. We are inclined to believe that the smaller irregularities in the diffusion rates found from day to day not only with glucose but also with the other substances are due to the unevenness of this process.

In summarizing, then, the points just discussed which have helped us form a picture of the structure of our membranes, we may conclude that the pore channels are of many different sizes. We know nothing of the number or importance of the pores smaller than the acetone molecule but have been able to estimate that of the remaining pore area only 2 per cent is distributed among pores large enough to allow the diffusion of glycerine and only 0.3 per cent among pores large enough for glucose.

In previous papers of this series (4, 9) we have dealt with the electrical effects demonstrable when the dried collodion membrane is placed between solutions of the same electrolyte in different concentrations. The $Co P$ value when a membrane is placed between 0.1 N and 0.01 N KCl solutions is the most familiar example. In a general way we have taken this $Co P$ value as a criterion of certain of the membrane properties and in particular have regarded it as giving some sort of an average estimate of the fineness of the pore channels. It would seem then that we should be able to draw more or less of a parallel between the $Co P$ values of our membranes and the ratios of the diffusion rates between molecules of different sizes, for we have regarded these ratios as depending upon the relative numbers of pores of larger and smaller size. In the early experiments of this study with acetone and glycerine we were disappointed in being able to find no such expected parallel. It is true that the membrane with the lowest $Co P$ value, F-6, also gave the lowest acetone-glycerine ratio but another membrane, C-6, with a moderately high $Co P$, showed almost the same ratio. Similarly the membrane F-5, with a $Co P$ value considerably below the average, gave the highest acetone-glycerine ratio of all. However, when one examines the figures secured from the experiments with glucose quite a different result is obtained. The glycerine-glucose, acetone-glucose, and for comparison the acetone-glycerine ratios with this series of membranes have been listed in Table IX, the membranes being arranged in order of

their *Co P* values. From this table it can be seen that no relation exists between *Co P* and the acetone-glycerine ratio,—that between the acetone-glucose ratio and *Co P* a suggestive relation exists in that only the two membranes with the highest and best maintained *Co P* values gave the very large ratios,—whereas between the glycerine-glucose ratio and *Co P* values with one exception a definite parallel can be drawn, the ratios increasing directly with the *Co P*. These ratios increase in a regular way from 1.46 for the membrane F-11 with

TABLE IX.

Membrane No.	<i>CoP</i> variations	Ratios of Diffusion Rates		
		$\frac{\text{Glycerine}}{\text{Glucose}}$	$\frac{\text{Acetone}}{\text{Glucose}}$	$\frac{\text{Acetone}}{\text{Glycerine}}$
	<i>mv.</i>			
F-2	28.1-22.2	2.09	115.8	55.4
F-11	32.1-25.7	1.46	64.2	43.8
C-6	41.1-35.9	4.18	99.4	23.8
F-8	47.0-40.3		45.4	
C-7	47.0-45.4	12.56	1075.8	85.7
C-1	49.7-49.0	45.45	1600.6	35.2

To show relation of *Co P* to various diffusion rate ratios. In this table the membranes studied with glucose have been arranged in order of their *Co P* values which are given with their variations during the period of study. In preparing the table several approximations were necessary. With the membranes C-1 and F-11 the acetone diffusion rate was calculated from the urea diffusion rate by multiplying the latter by 1.65 (average value for known acetone-urea ratio). With the membranes F-2, C-6, and C-7 the acetone and glycerine diffusion rates were determined at room temperature (about 23°C.); for comparison with glucose these rates were corrected for a temperature difference of 20°C. between the room and ice box by supposing the diffusion rates proportional to absolute temperature.

an average *Co P* of 28.9 millivolts to 45.45 for the membrane C-1 with an average *Co P* of 49.4 millivolts.

In order to harmonize and account for these apparently diverse results it is necessary to turn to a consideration of those factors in the membrane which are responsible for the potential difference arising when it is placed between solutions of an electrolyte in different concentrations. We have previously shown that this potential difference is dependent not only upon the ratio of the concentrations

of the electrolyte but also upon the particular concentration range being considered. In dilute solutions the effect of the membrane is marked and relatively high potential differences are observed whereas in more concentrated solutions the effect of the membrane tends to vanish. Moreover, we have shown that these effects can be satisfactorily explained by referring them to changes in the transfer number of the anion or more directly to changes in the mobility of the anion produced by the membrane. Thus when working with potassium chloride in high concentration the mobility of chlorine is almost as great as that of potassium and the observed potential effect is small. In low concentration the mobility of chlorine is small compared with that of potassium and a relatively large potential effect arises.

It now becomes of interest to search more closely for the factor responsible for the changes in the mobility of the anion with changes in concentration. It will be recalled that in any case the mobility is much less in the membrane than in free aqueous solution. We may suppose that this decrease in mobility is due to some sort of adhesion or adsorption between the anions and the boundary surface of the pore channels. When the concentration of the electrolyte is low enough there may be a channel surface great enough to bind all or nearly all of the anions migrating through the membrane. Similarly in a high concentration the channel surface may be great enough to bind only a small part of the migrating anions, the remainder moving with relative freedom through the channel lumens. We may thus refer the changes in the mobility of the anion to changes in the ratio between the number of anions in the solution and the area of the channel walls. With any membrane we can vary the first of these factors by changing the concentration of the electrolyte in the solution wetting it. The effect of varying the area of the channel walls cannot be observed in one membrane because this area is fixed. However, we can see in this factor an explanation for the fact that one membrane in a high range of concentrations may give the same potential difference as another membrane in a low range. Theoretically it is possible to conceive of a membrane with a constant pore-lumen area showing widely different potential effects depending upon whether

this area is made up of a few pores of relatively large size or many smaller pores.

In a previous paper we have shown that the curve expressing the dependence of the transfer number of the anion on concentration has a bilevel form.* In high and in low concentrations the dependence is slight; in a medium concentration range the dependence is great. Inasmuch as this bilevel curve is brought about when we vary in a regular way the ratio between the number of anions and the area of the channel walls by varying the concentration of the electrolyte we may infer that if we could vary in a regular way the size of the pore channels, the resulting curve expressing the transfer number of the anion would likewise have a bilevel shape for the effect on the determining ratio would be the same as produced by concentration changes. Thus we may suppose that there is a certain range of pore sizes through which the potential effects will vary widely. Pores of smaller size than those included in the range will have little more effect than if they were large enough to be included in the smaller end of the range, and similarly the effect of increasing the size of a pore will be slight after this range is surpassed.

Now, in the case of non-electrolytes diffusing through the membrane, there are no adhesive forces from the cell walls such as are present with an electrically charged ion. The rate of passage depends merely upon the number of pores large enough to transmit them. We have here then a rational explanation for the seemingly diverse results obtained in our diffusion experiments. We may imagine that the majority of the pores which allow the passage of acetone and glycerine are too small to be included in the range responsible for marked potential variations. The ratio of the glycerine rate to the acetone rate would then tell us nothing of the electrical effect to be expected. On

* As curves of this shape are encountered very often in biochemical investigations (dissociation curves, titration curves, etc.) we have introduced the descriptive term "bilevel" by which they may be designated. Such a curve may be defined as one in which the dependent variable depends on the independent to an appreciable extent only within a limited range beyond which the curve flattens on both sides to two parallel asymptotes each at a different level. Moreover, the curve has only one inflection point which lies in the variable part of the curve and the sign of the first derivative never changes. Such a curve is represented in Chart 2.

the other hand it is extremely likely that the pore sizes between the size of the glycerine and glucose molecules include at least a part of this range for here there is an evident relation between the diffusion rate ratios and the $Co P$ values.

It may be helpful to represent the conception embodied in the foregoing discussion in a schematic way (Chart 2). Here we have set S_1, S_2, S_3 , etc. to stand for the pores of increasing size in a membrane and N_1, N_2, N_3 , to represent the number of pores of each respective size. Then the entire pore system of the membrane can be represented by $N_1S_1 + N_2S_2 + N_3S_3$, etc. The curve shows the manner in which

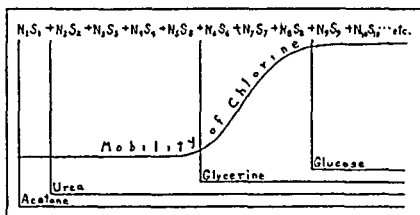


CHART 2. Schematic representation of the pore system of the dried collodion membrane. S_1, S_2, S_3 , etc. refer to different and increasing pore sizes; N_1, N_2, N_3 , etc. indicate the number of pores of each size. The bilevel curve shows an hypothetical zone in which anion mobility varies greatly with increasing pore size. The possible pore sizes concerned in the diffusion of the various non-electrolytes are indicated in the lower part of the diagram.

the mobility of the anion and therefore the $Co P$ value may depend upon the various pore sizes. The lower part of the chart shows the pore sizes able to function in the diffusion of acetone, urea, glycerine, and glucose. An inspection of this chart shows that countless arrangements of pore sizes and numbers are possible but that only when these fall within a certain range will there be any effect on the $Co P$.

We are aware that the foregoing theory offers no explanation for the fact that the acetone-glucose ratio does not correspond with the $Co P$ value as well as the glycerine-glucose ratio. Referring again to Chart 2 it will be seen that both of these ratios cover the 1

range of electrical importance. As yet we do not feel prepared to offer an adequate explanation for this discrepancy. We may state, however, that several other considerations have rendered plausible the idea that in a certain range of pore sizes there may be an effect on cation mobility as well as anion mobility and that by a development of this idea it is possible to account for the discrepancy just mentioned. However, we shall not feel justified in developing this idea before definite experimental data on this point has been collected.

SUMMARY.

A study has been made of the relative rates at which various organic non-electrolytes diffuse through the dried collodion membrane. It was found that acetone and urea pass through the membrane many times more rapidly than glycerine and that glycerine in its turn diffuses much faster than glucose. It was also demonstrated that the rate of diffusion varies directly with the difference in concentration between the solutions on the two sides of the membrane. It was shown that the presence of glycerine on the two sides of the membrane did not appreciably affect the rate of diffusion of acetone. In a study of the changes going on during the establishment of the stationary diffusion gradient with glucose experiments were described which strongly suggested that many of the membrane channels may gradually become clogged up with glucose molecules so that the diffusion rate decreases from day to day until the stationary gradient is finally reached.

In explaining the various experimental data the conception of the collodion membrane as a sieve with pores approximating in smallness the size of individual molecules was utilized. The large differences in the diffusion rates between different substances were then referred to differences in molecular size, the relatively large molecules of glycerine and glucose being unable to pass through many of the smaller pores available for urea and acetone. From the data available it was possible to estimate that 98 per cent of the pore area distributed among holes large enough for the diffusion of acetone was unavailable for the passage of glycerine and that only 0.3 per cent of the pore area available for acetone could be utilized by glucose. In trying to correlate the ratio between the diffusion rates of two different

substances with the characteristic concentration potential ($CO P$) given by the same membrane it was found (1) that with the acetone-glycerine ratio there is no correspondence (2) that with the acetone-glucose ratio a suggestive relation exists and (3) that with the glycerine-glucose ratio a definite correspondence can be shown, the higher ratios being obtained only with membranes giving high $CO P$ values. A rational explanation for these facts was proposed.

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DARK ADAPTATION IN AGRIOLIMAX.

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I.

When the slug *Agriolimax*, previously dark adapted, is allowed to creep geotropically upward upon a vertical plate, lateral illumination forces it to pursue a path at an angle (β) to the perpendicular (*cf.* Wolf, 1926-27) and away from the light. The magnitude of this angle decreases with time (Wolf and Crozier, 1927-28), due to photic adaptation in the eye (Crozier and Wolf, 1927-28). The change with time during continuous exposure is adequately accounted for, quantitatively and very simply, by the assumption that the path at any moment is the resultant of 2 vectors, respectively those determined by geotropic and by phototropic excitation, and that these vectors express quantitatively the effects of the corresponding excitations (Wolf and Crozier, 1927-28). The geotropic excitation is taken to be constant, while the phototropic excitation decreases exponentially with time, due to the first order character of the light adaptation. These assumptions lead to an expression for β as a function of time,

$$\text{time} = a - \frac{1}{K} \log \tan \beta, \quad (1)$$

which is very satisfactorily obeyed; and to the expectation that the rate of adaptation, K in equation (1), should be practically a logarithmic function of the light intensity (over intermediate ranges), which is also substantiated. The assumptions underlying this treatment are that the intensity of excitation is proportional to $-\frac{ds}{dt}$, where S is the substance which is decomposed photochemically,

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and that with the animals fully dark adapted at the start — $\frac{ds}{dt}$ is *effectively* proportional to $[S]$ at each moment, any reverse reaction serving to regenerate S being negligible under the conditions (at lower temperatures, when dark adaptation is known to be quite slow). It was pointed out (Wolf and Crozier, 1927–28) that this type of experiment can be reversed for the study of dark adaptation. It is important to determine the characteristics of the receptor mechanisms concerned with continuous excitation by light, as in phototropism, and we have accordingly made experiments regarding the kinetics of dark adaptation in *Agriolimax*. The continuous nature of the photic excitation is proved by the character of the path of orientation. The orientation is fully accounted for by the assumption that the difference in tonus of the musculature which results in turning is proportional to the photic excitation. The results demonstrate one further respect in which the receptor machinery for phototropic excitation and that for differential sensitivity, as studied by Hecht (1918–19; 1926–27) in several forms, are fundamentally akin. They may also be invoked as supplying an additional chapter in the development of the prediction of conduct in compound fields of stimulation (Crozier, 1923–24; 1926–27; Crozier and Pincus, 1926–27; Crozier and Stier, 1927–28).

II.

Agriolimax was allowed to become light-adapted by exposure for at least 2 hours to bright daylight at a window facing an unclouded sky. It is necessary to make sure at intervals that the slugs are creeping, with eye-stalks everted. Exposure to direct sunlight is quickly injurious, but illuminations of about 1,000 m. c. proved non-toxic and adequate (*i.e.*, sufficiently high) for our purpose. Light-adapted individuals were then taken in succession and placed in darkness for 5 minutes each. A curve of light adaptation was then obtained for each, by measuring the divergence from perpendicular creeping upon a moist vertical plate of ground glass, as earlier described (Wolf and Crozier, 1927–28). The adapting light was of 29.4 f. c. The data from a sample of 10 individuals treated in this way show good agreement, and the readings of the angle of divergence (β) at successive minutes during light adaptation are accordingly averaged. The

TABLE I.

Amplitudes of response (β) at successive intervals in lateral light of 29.4 f.c. intensity, beginning at various levels of dark adaptation. (The magnitudes of β have been checked by further groups of experiments.)

Time in darkness	No. of individuals	Time in light	β
<i>min.</i>		<i>min.</i>	
5	16	0.5	$31.98^\circ \pm 0.995$
		1.5	$21.23^\circ \pm 1.00$
		2.5	$13.07^\circ \pm 1.02$
10	15	0.5	$40.28^\circ \pm 1.208$
		1.5	$26.34^\circ \pm 0.605$
		2.5	$18.97^\circ \pm 0.477$
		3.5	$12.62^\circ \pm 0.639$
15	17	0.5	$44.06^\circ \pm 1.05$
		1.5	$31.87^\circ \pm 0.702$
		2.5	$25.0^\circ \pm 0.838$
		3.5	$18.0^\circ \pm 0.526$
20	10	0.5	$59.45^\circ \pm 1.25$
		1.5	$45.80^\circ \pm 1.28$
		2.5	$34.30^\circ \pm 1.20$
		3.5	$25.80^\circ \pm 1.02$
25	8	0.5	$58.0^\circ \pm 1.25$
		1.5	$46.63^\circ \pm 1.14$
		2.5	$35.81^\circ \pm 0.940$
		3.5	$26.44^\circ \pm 1.05$
30	10	0.5	$59.25^\circ \pm 1.97$
		1.5	$42.60^\circ \pm 1.30$
		2.5	$30.25^\circ \pm 1.45$
		3.5	$20.90^\circ \pm 1.24$
45	17	0.5	$61.60^\circ \pm 1.05$
		1.5	$49.90^\circ \pm 1.17$
		2.5	$34.62^\circ \pm 0.723$
		3.5	$25.43^\circ \pm 0.821$
60	14	0.5	$62.50^\circ \pm 1.75$
		1.5	$47.2^\circ \pm 0.936$
		2.5	$34.33^\circ \pm 1.03$
		3.5	$24.89^\circ \pm 0.529$

tests are then repeated with similar slugs each dark-adapted for 10 minutes, and so on for succeeding intervals up to 60 minutes (Table I). The temperature in the dark room was held at 20.5° to 22.0° throughout. At this temperature the rate of increasing effectiveness of photic excitation is still very slow by comparison with that of adaptation by light.

It is impossible to carry through such series of observations, or rather to interpret the results in a simple and direct way, when single individuals are tested at different times during their dark adaptation (as subsequently discussed), and physical limitations make it necessary to deal with relatively small groups of individuals at a time (their respective intervals in darkness being properly spaced and controlled). The data for each curve of light adaptation has therefore been based, as a rule, upon a group of ten slugs. Individual differences, of quantitatively perceptible order but of consistent sense, expose the interpretation of such data to certain hazards. At each desired point on the final curve of dark adaptation, therefore, the tests were repeated with new groups of individuals. Aside from possible but slight variations in the intensity of the test light, and of the adapting illumination, the existence of diurnal variations in photic excitability must be guarded against; and, more important in a practical way, diurnal fluctuations in geotropic responsiveness. It is known, from our own experience with *Agriolimax* and other slugs, that the geotropic response is more powerfully in evidence, or—what may not be the same thing—more frequently expressed,—toward evening and after night-fall than in the early morning hours. Diurnal fluctuations of activity have long been noted in other forms. In our earlier work such changes have been discounted by running the experiments between hours 11 a.m. and 6 p.m., and by using a large number of series of observations spread more or less uniformly over this interval.

The derivation of equation (1) assumed that g , the geotropic vector, remained constant. It is also assumed that the threshold value of the photic excitation, required to produce any phototropic orientation on the vertical plate, is not a value to be subtracted from the total photic excitation, but that if effective at all the photic stimulation has its full proportionate value. Questions concerning the central nervous interplay between photic and gravitational stimulations receive some

further elucidation in experiments subsequently made with *Limax*, to be treated in another place. Variations in this threshold quantity would not affect the intercept on the t axis in Fig. 1, nor the slope of the curve of light adaptation.

We have been fully alive to the fact that in treating the photic excitation no account has been taken of the inclination of the receptive surface to the path of the light. One can suspect that change in this angle would alter the magnitude of our L . But the question is complicated by the fact that the eye-tentacles of *Agriolimax* diverge further as light adaptation increases, so that the angle between the eye-surfaces ("H" of a previous paper—Crozier, 1925-28) decreases as the slug comes to orient more nearly straight upward. This would tend to increase L illegitimately, and to cause curves such as those in Fig. 1 to be *slightly* concave upward. In a number of instances this appears to be true, and in no instance is the reverse true. But the effect, if real, is of small magnitude, with the orienting light of moderate intensity; and it may possibly be accounted for in other ways (cf. § VII).

III.

The notion that excitation by light is a photochemical matter, and that the maintenance of photic reactivity and its recovery after exhaustion may be in some fashion connected with a reversible photochemical receptor system seems to have been held in a very general form since Hering. Growing knowledge of the chemical effects of light made it attractive to translate such ideas into photochemical terms. An early attempt to treat the question precisely and specifically, but with some important deficiencies, was made by Müller (1896). To explain the simultaneous exhibition of capacity to react to a sharp decrease in light intensity and also to be excited to phototropic movements by the continuous action of light, as seen in the integument of certain holothurians, it was suggested that the same photochemical system, if part of a reversible reaction of a certain kind, might be conceived to serve for both modes of stimulation (Crozier, 1915). Hecht¹ has been able to achieve the step-by-step proof, in a very satisfying and convincing manner, that a particular

¹ Hecht, 1918-19; 1919-20, *a*, *b*; 1922-23; 1925-28; 1926-27.

type of reversible photochemical system underlies photic excitation of *Mya*, *Ciona*, *Pholas*, and the human eye. The properties of this system permit the prediction and the rational quantitative interpretation of new and unexpected characteristics of the stimulation process. A major property of the underlying mechanism is adequately expressed (Hecht, 1918-19; 1922-23) in the following paradigm, where *S* represents primary photosensitive substance, *P* and *A* two of the products of its photolysis:



The system is reversible, and with time, under continuous constant illumination, there is established a stationary state ("equilibrium"). The "dark" process, of which the kinetics serves to describe the course of dark adaptation, is definitely bimolecular (Hecht, 1926-27), with high temperature characteristic; the "light" reaction is of first order and negligibly influenced by temperature (Hecht, 1919-20, *a*).

These relationships have been established most elaborately by means of experiments with organisms in which the speed of response following the delivery of a measurable quantity of light gives data necessary for the quantitative treatment, and in which light adaptation is rapid and photic excitation therefore necessarily discontinuous. To carry over such considerations to the analysis of continuous excitation, as in phototropic stimulation, it has been necessary to find phototropic organisms in which the rate of light adaptation is measurable, neither inconveniently rapid nor too slow. The photic adaptation of *Agriolimax* has been followed by causing the phototropic influence of the light to work against the "brake" provided by a vectorially constant excitation of a different kind, namely that due to gravity, acting at right angles to the phototropic vector. The function of such a "brake" in contributing to the significance of the measured orientations has been discussed previously in relation to circus movements (Crozier and Federighi, 1924-25). Data necessary for treatment of the light adaptation are gotten from measurements of the resultant angles of orientation as related to time and to intensity (Wolf and Crozier, 1927-28). Such measurements are possible because the rate of light adaptation, especially at temperatures in the neighborhood of 15° or slightly above, is so very much faster than

that of dark adaptation, as adequate tests showed. It was easily established that the rate of light adaptation is very little influenced, if at all perceptibly, by temperature; and that its course follows that of a first order reaction in which the "velocity constant" is a linear function of the logarithms of the light intensity. These phenomena therefore parallel in an exact manner the properties of the "light" reaction already discussed.

The interpretation of dark adaptation requires measurements of the photic excitability at successive intervals following exclusion of light from individuals previously light-adapted. To reduce the probability of adventitious errors of estimation the experiments were made at a temperature high enough to bring the time for practically complete dark adaptation down to a little over 1 hour. At about 12°, the time required may be as long as 3 to 4 hours; at 20.5°-22°, the temperature prevailing throughout the present experiments, the time is less than half of this. Since light adaptation, *as measured by the power to influence geotropic creeping on a vertical surface*, is effectively complete within about 5 minutes, as a rule, with high intensities, the experiments must, for simplicity, take the form of establishing curves of light adaptation as affected by known preceding periods in darkness. From such data it is required to deduce the ideal photic excitability at the last moment in darkness, before the exposure has begun, or in other words the kinetics of the "dark" reaction. The necessity for speedy and precise manipulation has made it undesirable, for the present, to vary the light intensity from moment to moment in such a way as to keep the angle β constant. This alternative method has certain theoretical advantages, however, and subsequently will be used!

IV.

Two aspects of the measurements at once present themselves—the *rates* of light adaptation after various periods in the dark, and the magnitudes of β at the first instant of exposure to the light. It is apparent from Fig. 1 that the course of light adaptation after given conditions of adjustment in the dark adheres to the equation previously obtained,

$$\text{time} = a - \frac{1}{K} \log_{10} \tan \beta,$$

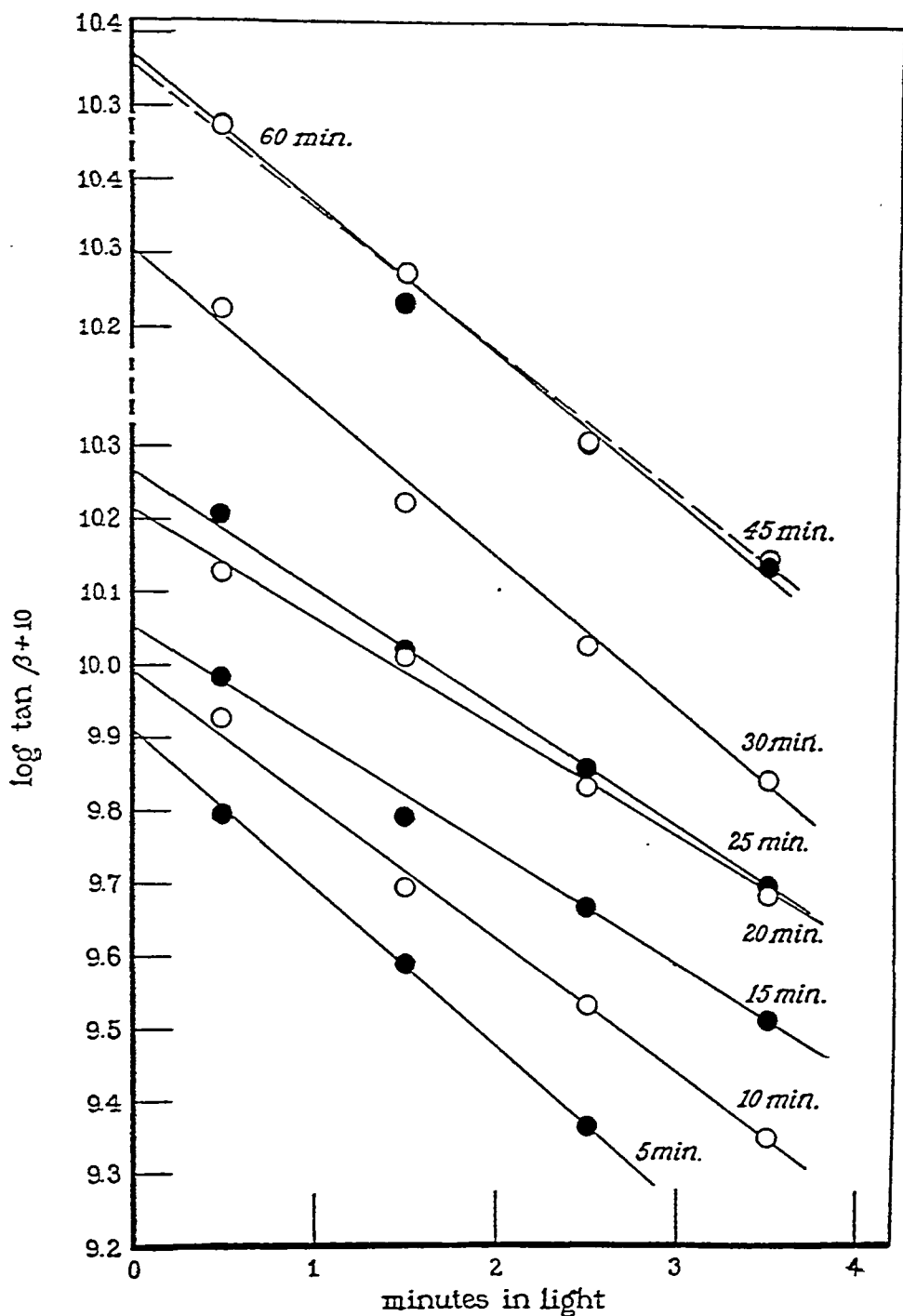


FIG. 1. The angle (β) of orientation of *Agriolimax* away from the vertical path on a perpendicular plate, as enforced by exposure to horizontal light of 29.4 f.c. on the right side, at successive minutes during the exposure, following increasing residence in darkness (5 to 60 minutes) of slugs initially light-adapted. Time of exposure plotted against $\log \tan \beta$ gives graphs sensibly rectilinear. The intercepts of these graphs on the ordinate axis give a means of estimating the photic excitability at the very first moment of exposure to light (see text). (The ordinate axis has been shifted vertically, for "30 min." and for "45 min." and "60 min.," to obviate overlapping.)

and that K , the rate coefficient of light adaptation, and a , the intercept on the β axis corresponding to zero time of exposure to light, each vary with the extent of dark adaptation. From the assumptions made and tested in the previous papers, we may write

$$\tan \beta = \frac{L}{g}, \quad (3)$$

where L and g represent respectively the photic and the gravitational orientation vectors. If g is constant, $L = g \tan \beta$. L is taken to be a measure of the average amount of photosensory substance present,

TABLE II.

Intercepts on the $\log_{10} \tan \beta$ axis (*i.e.*, $\log_{10} \tan \beta_i$) at $t = 0$ exposure to light (29.4 f.c.), following increasing residence (t) in darkness of initially light-adapted slugs. $R = \frac{KL_\infty}{KL_i}$; KL_∞ = intercept for completely dark-adapted animals.

t	$\log_{10} \tan \beta_i$	β_i	R
<i>min.</i>			
5	9.910-10	39.1°	3.020
10	9.996	44.7°	2.477
15	10.059	48.9°	2.143
20	10.216	58.7°	1.528
25	10.264	61.5°	1.337
30	10.305	63.7°	1.216
45	10.350	65.9°	1.096
60	10.362	66.5°	1.067
∞	10.390	67.9°	1.000

S , or more precisely as the average value of $-\frac{ds}{dt}$, during the interval over which β is estimated. This is the amount of substance the increase of which, in terms of the conception of a reversible photochemical system, we desire to follow during dark adaptation. The two assumptions as to the nature of L will be tested separately.

Taking the intercepts on the $\log_{10} \tan \beta$ axis of Fig. 1, and from these obtaining $\tan \beta$, we have numbers proportional to the concentrations of S present at the beginning of the test-exposures, if the "dark" reaction is relatively negligible; this follows from the primary assump-

tion (Wolf and Crozier, 1927-28) that the phototropic excitation in any short interval is proportional to $-\frac{ds}{dt}$, and thus to the amount of S photolysed in unit time. Strictly speaking, this of course may not be entirely accurate, since the angle β is estimated over a finite length of time and of path; but deficiencies arising in this way are lost among the individual variations of response and are of no moment

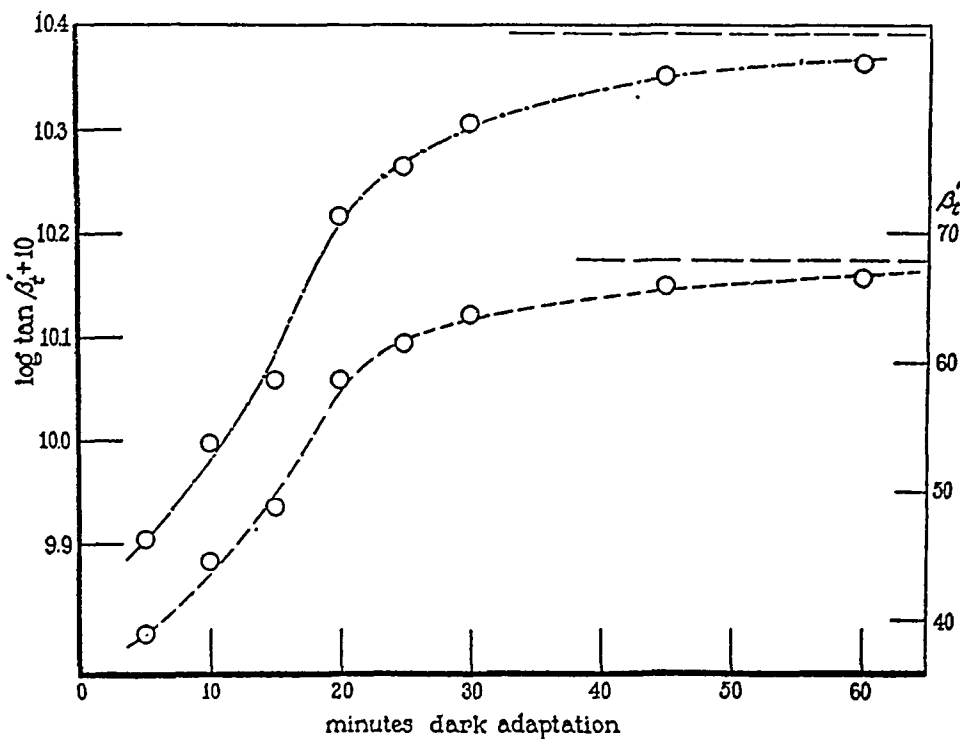


FIG. 2. Curves showing the increase of the intercept angle (β'_t), and of $\log \tan \beta'_t$, as a function of time in the dark subsequent to prolonged exposure to light.

for our present purpose. The values of $\tan \beta$ corresponding to $t = 0$ increase regularly with time of residence in darkness (Table II and Fig. 2), and the adaptation is therefore an orderly process. The type of reaction which describes the kinetics of dark adaptation, and thus of the "dark" reaction in the absence of light, may be ascertained from the data in Table I and Fig. 2. Since $\tan \beta$ is proportional to L , the intensity of excitation, and this by assumption to $-\frac{ds}{dt}$ and

so to S_t , the amount of photosensitive substance present at the moment, we might write

$$\frac{\tan \beta_{\infty}}{\tan \beta_t} = \frac{L_{\infty}}{L_t} = R, \quad (4)$$

where β_t is the angle given by the intercept on the $\log \tan \beta$ axis in Fig. 1, —i.e., corresponding to zero time in the light, after t minutes dark adaptation, and β_{∞} is the intercept angle after complete dark adaptation. Thus we have a measure of the photic sensitivity at the first moment of exposure to the test light. From (4), since $L_t \propto S_t$, S_t being the concentration of S after time t in the dark,

$$\frac{[S_{\infty}]}{[S_t]} = R; \quad (5)$$

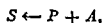
$\tan \beta_{\infty}$ is obtained from observations on animals fully dark-adapted (Wolf and Crozier, 1927–28).

If the reformation of S in the dark were a first order process, $\log S_t$ should increase linearly with time. We do not know S_t in absolute units, but we have a proportionate measure of it from (4) and (5).

More precisely, if we assume $R = \frac{dx'/dt}{dx_t/dt}$, where dx'/dt = the rate of photolysis of S when dark adaptation is at first *complete*, and therefore $\propto [S_{\infty}]$, then $-\frac{dS_t}{dt} = \frac{dx_t}{dt} = \frac{kS_{\infty}}{R}$; this, on the assumption of the

moment, would be equal to $K_2 (S_{\infty} - S_t)$, and $\log \frac{S_{\infty}}{R}$, or $\log R$, should still be a linear function of time in the dark. The assumption of a “monomolecular” formation of S can therefore be tested by plotting $\log \frac{S_{\infty}}{R}$, or $\log R$, against time of dark adaptation. As Fig. 3 shows, the relationship is certainly not rectilinear, and this assumption may be dismissed; moreover, it would leave unexplained the changes in *slope* of the curves of light adaptation.

If the formation of S in the dark be assumed bimolecular, corresponding to



then $(S_{\infty} - S_t)$ must decrease hyperbolically with time in the dark,

or the graph of $1/(S_{\infty} - S_t)$ vs. t must be linear; but $S_{\infty} - S_t = (R - 1)(S_t) = \frac{(R - 1)(S_{\infty})}{R}$, and therefore $\frac{1}{S_{\infty} - S_t} = \frac{1}{S_{\infty}} \cdot \frac{R}{(R - 1)}$; since $\frac{1}{S_{\infty}}$ is by assumption constant, the plot of $\frac{R}{(R - 1)}$ against time in

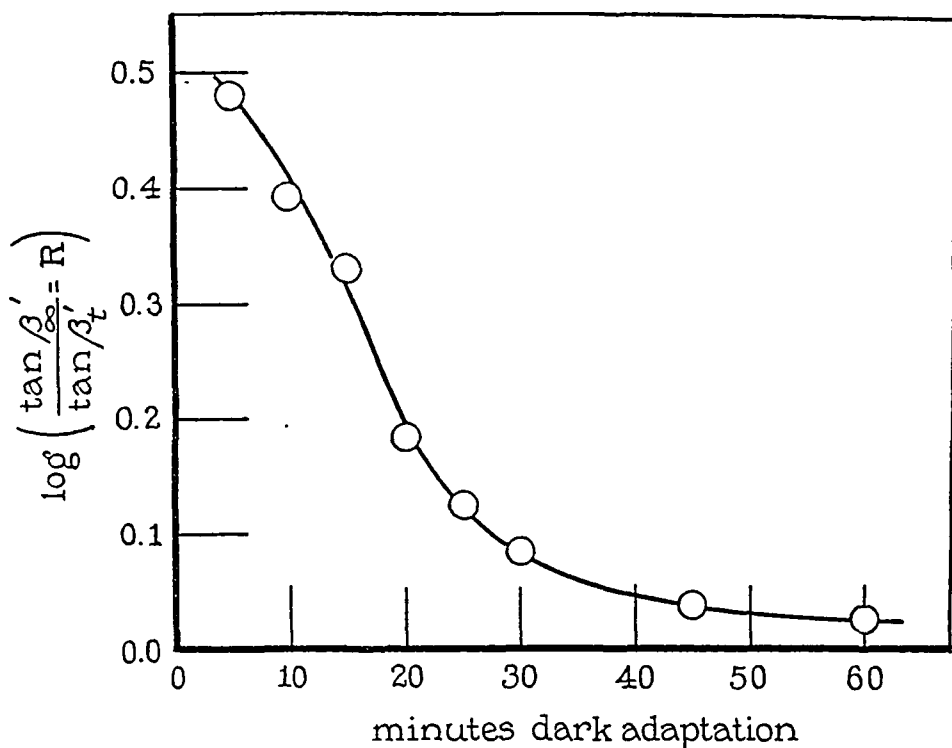


FIG. 3. If the course of dark adaptation were describable as a first order process $\log R$ should be linearly proportional to time in the dark,—which is obviously not the fact. R is the ratio between initial excitability after complete dark adaptation and that after t minutes in darkness. The excitability may be taken as proportional to $[S]$, the concentration of photosensitive substance at the moment, or to $-\frac{ds}{dt}$.

darkness should show a linear increase. As is evident in Fig. 4, the relationship is anything but linear; in fact the curve shows a distinct sigmoid flexure and apparent relative inhibition of formation of S at the start. If we prefer to regard L as proportional to $-\frac{ds}{dt}$, then it is

easily shown that R , for a second order process, should be linearly proportional to t —which is certainly not the fact.

To account for the form of this curve it is necessary, as the simplest assumption, to invoke some type of autocatalysis. If the "dark" reaction were first order and autocatalyzed by S , then

$$-\frac{\delta x}{\delta t} = K_2 (S_\infty - x) (x) + K'x \quad (6)$$

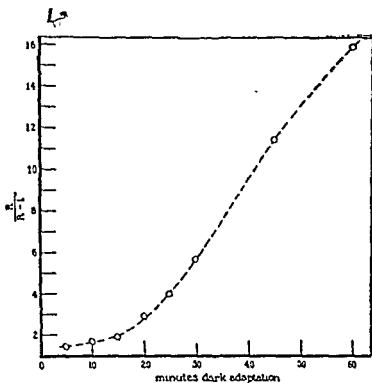


FIG. 4. Were the course of dark adaptation susceptible to formulation as a second order reaction, then $\frac{R}{R-1}$ should be a rectilinear function of confinement to darkness. Clearly, such formulation fails.

where x = concentration of A and of P ; during exposure to light, also

$$\frac{\delta x}{\delta t} = K_2 (S_\infty - x), \text{ and}$$

$$\frac{dx}{dt} = K_2 (S_\infty - x) (x) + K'x - K_1 (S_\infty - x) = (K_2 x - K_1) (S_\infty - x) + K'x \quad (7)$$

If for simplicity we assume, as has usually been done in the treatment of first order autocatalysis by a reaction product (*cf.* Crozier, 1926-27).

that K' is negligibly small, then the course of dark adaptation (K_1 being then $\equiv 0$) is given by

$$\frac{\delta x}{\delta t} = K_2 (S_{\infty} - x) (x); \quad (8)$$

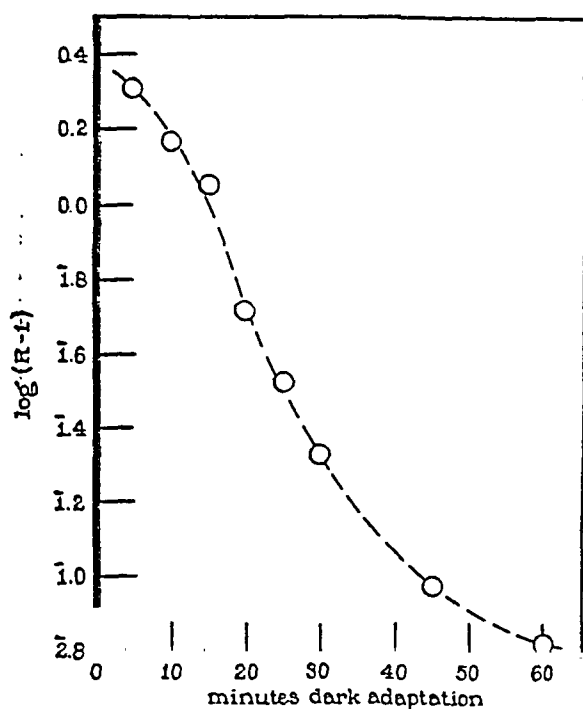


FIG. 5. The "dark" formation of photosensitive substance cannot be accounted for as the result of a first order process with positive autocatalysis, since $\log \frac{x}{S_{\infty} - x}$ (or its proportionate equivalent, $\log(R - 1)$), is not a linear function of time in the dark.

and, as is well known, the graph of $\log \frac{x}{S_{\infty} - x}$ against time should show a linear decrease (if the value of S_{∞} is properly chosen); in terms of our present notation, from (4), $\log \frac{x}{S_{\infty} - x} \equiv \log(R - 1)$.

It is clear from Fig. 5 that the graph is not rectilinear; nor is its approach to rectilinearity at all improved by the assumption of other

(slightly different) magnitudes for S_{∞} . Moreover, even assuming for a proposed autocatalytic effect of first order type the more complete differential equation

$$\frac{\delta x}{\delta t} = K_1 (S_{\infty} - x) (x) + K'x,$$

the inflection point in the sigmoid curve relating S to t during dark adaptation must come at

$$\frac{d^2 S}{dt^2} = K_2 S_{\infty} - 2K_2 x + K' = 0,$$

whence $x = \frac{K_2 S_{\infty} + K'}{2K_2}$; in other words, the inflection must come at a point such that S is less than $\frac{1}{2} S_{\infty}$ (although it might be very close to $S = \frac{1}{2} S_{\infty}$); but this quite disagrees with the facts (*cf.* Fig. 4, and Fig. 6). In terms of the more rigorous assumption that $L \propto \frac{ds}{dt}$, the idea of first order autocatalysis is even more poorly substantiated.

V.

We must therefore give up a conception of first order formation of S during dark adaptation, and turn to a second order process (with auto-acceleration by S) as possibly giving the simplest description of the facts. The integral equations for such processes are not easy to handle, and indeed must be devised for the occasion; but they provide several decisive tests which are of considerable interest. These tests are of a nature which tends to remove one's suspicion that the agreement between formula and experiment might be merely a descriptively successful accident.

We will assume that the differential expression for the second order autocatalysis may be written

$$\frac{ds}{dt} = K_1(S) (x^2) = K_2 (S_{\infty} - x) (x^2),$$

neglecting, as before, the additional catalytic effect more properly

cared for by the introduction of another velocity constant. Then for the rate of change of $[S]$ in the light we will have

$$-\frac{\delta s}{\delta t} = K_1 (S_{\infty} - x),$$

and for the dark reaction

$$\frac{\delta s}{\delta t} = K_2 (S_{\infty} - x) (x^2),$$

and therefore

$$\frac{ds}{dt} = K_2 (S_{\infty} - x) (x^2) - K_1 (S_{\infty} - x), \quad (9)$$

or

$$\frac{dx}{dt} = -\frac{ds}{dt} = K_1 S_{\infty} - K_1 x - K_2 S_{\infty} x^2 + K_2 x^3.$$

When x is very small (relatively complete dark adaptation) $-\frac{ds}{dt}$ on exposure to light will thus depend largely on $K_1 (S_{\infty} - x)$, and (other things equal) will be large (*cf.*, Wolf and Crozier, 1927-28); when x is large $-\frac{ds}{dt}$ will be low and its rate of change in light will be slowed, roughly in proportion to x^2 . This corresponds in a crude way to the increase in the slope of the curve of light adaptation as dark adaptation proceeds (Fig. 1).

The curve describing the change of photic excitability with increasing dark adaptation is given, on this assumption, by

$$\begin{aligned} y = -\frac{ds}{dt} &= K_1 S_t - K_2 (S_t) (S_{\infty} - S_t)^2, \\ &= K_1 S_t - K_2 S_{\infty}^2 S_t + 2 K_2 S_{\infty} S_t^2 - K_2 S_t^3, \end{aligned} \quad (10)$$

where $y, = -\frac{ds}{dt}$, is proportional to L at the first instant of exposure to the light, and so, as before, to $1/R$. As $[S]$ increases, $\frac{dy}{ds}$ is the slope of $1/R$ plotted against S as S increases during the time of dark adapta-

tion. The slope of $1/R$ passes through an inflection point, since dy/ds is maximum at

$$d^2y/ds^2 = 0 = -4 K_2 S_\infty + 6 K_2 S, \text{ or } S = \frac{2}{3} S_\infty,$$

whence

$$ds/dt = \frac{2}{3} \frac{ds_\infty}{dt}, ds/dt / ds_\infty/dt = 1/R = \frac{2}{3}.$$

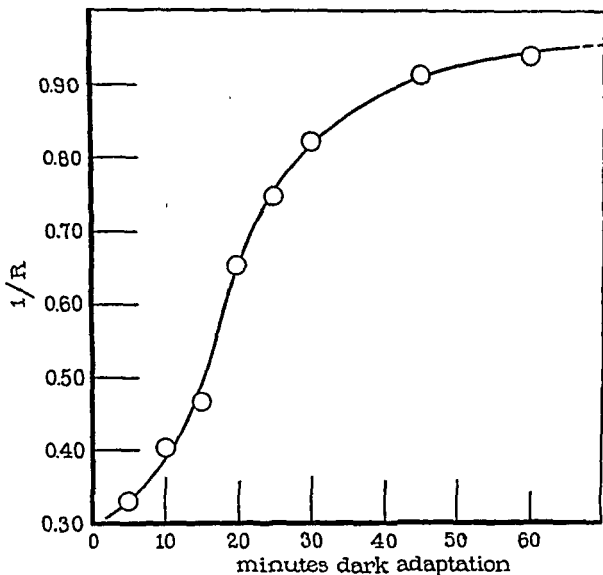


FIG. 6. The "dark" restitution of S through a second order reaction with positive autocatalysis would require that the photic excitability, $-\frac{ds}{dt}$, or its proportionate equivalent $1/R$, should exhibit a maximum velocity of change when $[S_t]$ has been brought to a little less than $\frac{2}{3} [S_\infty]$; $1/R$ should therefore pass through an inflection point when $1/R =$ a little less than 0.67—; the inflection is found at $1/R = 0.64$.

If the suppressed velocity constant K' be taken into account, x at the inflection point $= \frac{1}{3} \left(\frac{K'}{K_2} + S_\infty \right)$ and $\frac{1}{R}$ must be a little less than $\frac{2}{3}$. This graph is given in Fig. 6. The general form of the curve is as expected, and the empirically determined inflection point is at about 0.63 or slightly more. The agreement is therefore strikingly good.

It might be said that the most sensible thing would be for the animal to be *most rapidly* dark-adapting when its receptors are most completely exhausted by light. But the natural behavior of these slugs is of course such that they are probably never driven normally to so complete a level of light adaptation as that involved in these experiments. Their negative heliotropism serves to carry them into dark places at sunrise, so that the most rapid phase of their dark adaptation in all probability corresponds to the maximum degree of light adaptation which they are called upon to experience. Even after hours of exposure to daylight (not direct sunlight) they are still negatively phototropic, but in a feeble way.

The sigmoid character of the course of dark adaptation is also made evident when the slugs are tested *once* after a measured period in darkness subsequent to light adaptation. The angles β are thus obtained for the first minute of exposure to light. Owing to the manner in which both intercept and slope of the lines in Fig. 1 change, the inflection must come earlier in the curve than relating $\tan \beta_1$ to time in darkness than in the curve in Fig. 6. The data for an experiment of this sort given in Fig. 7 are somewhat scattered, but the inflection is at about 15 minutes and thus definitely earlier than in Fig. 6.

The inflection point in Fig. 6 comes at about $1/R = 0.63$, which corresponds to $\beta = 44.5^\circ$. It would be reasonable then, to expect a greater *variability* in the observed values of β at about this magnitude of the orientation angle, since at this level of adaptation, with test light of this intensity, $-\frac{ds}{dt}$ is changing most rapidly. Inspection of the probable errors (Table I) shows that the p. e. declines as β becomes less, but there are few measurements above $\beta = 45^\circ$; in earlier series (Wolf and Crozier, 1927-28) the effect predicted is reasonably clear.

VI.

The integral for the curve of dark adaptation is obtainable only in an inconvenient form. Assuming that $R = \frac{S_\infty}{S_t}$

$$\frac{ds}{dt} = K_2 S_t (S_\infty - S_t)^2 = \frac{K_2 S_\infty^3}{R^3} (R - 1)^2 = -S_\infty R^{-3} \frac{dR}{dt},$$

therefore

$$-\frac{dR}{dt} = \frac{K_2 S_\infty^3}{R} (R - 1)^2$$

and

$$-K_2 S_\infty^3 t = \int \frac{R dR}{(R - 1)^2} = \log (R - 1) - \frac{1}{(R - 1)} + C,$$

or

$$K_2 S_\infty^3 (t_1 - t_2) = \frac{R_2 - R_1}{(R_1 - 1)(R_2 - 1)} + \log \frac{(R_1 - 1)}{(R_2 - 1)} \quad (11)$$

This enables us to evaluate K_2 from the directly measured magnitudes of R . The calculated values of K_2 , as obtained from equation (11) and the tabulated values of R (Table II), taking $t_1 = 60$ minutes dark adaptation, show satisfactory constancy (Table III). This reference-point is not the most accurately established, but permits computing from the most points. Values of R read from the smooth curve (Fig. 6) give better constancy for K'' . Slight errors in obtaining R affect the computed value of K'' considerably. The systematic rise in K'' during the first 15 minutes in darkness might be explicable as due to the failure to consider the suppressed velocity constant in the autocatalytic formula (this makes $\frac{ds}{dt}$, hence $\frac{1}{R}$, appear "large"). Moreover, and this is most important, the derivation has assumed that the "dark" reaction is insignificant even at low concentrations of S , which is inadequate, and results in K'' passing through a maximum.

The complete expression for the rate of light adaptation is even more unmanageable. But several of its properties may be deduced, which

is all that is necessary for our purpose. If the conceptions underlying the analysis are correct, we must expect the rate of light adaptation to be a minimum when the rate of "dark" formation of S is highest. The change in the slopes of the curves of light adaptation (Fig. 1)

TABLE III.

Values of the velocity constant for dark adaptation, from $K_2 S_\infty^2 (t_1 - t_2) = \frac{R_2 - R_1}{(R_1 - 1)(R_2 - 1)} + \log \frac{(R_1 - 1)}{(R_2 - 1)}$, using ascertained values of R [Table II], taking $t_1 = 60$ minutes, and assuming $R = S_\infty/S_t$ (see text).

$t_1 - t_2$	$K_2 S_\infty^2 = K''$
<i>min.</i>	
15	0.320
30	0.341
35	0.358
40	0.314
45	0.294
50	0.267
55	0.241

should therefore supply an independent check on the interpretation. From equation (6),

$$-\frac{ds}{dt} = K_1 S_\infty - K_1 x - K_2 S_\infty x^2 + K_2 x^3;$$

this should give a clue to singularities in the curve relating $-\frac{ds}{dt}$ to changes in the concentration of x , for which the slope is

$$-\frac{d^2s}{dx^2} = -K_1 - 2K_2 S_\infty x + 3K_2 x^2 \quad (12)$$

For a maximum or minimum the next differential must be equatable to zero, and for a minimum $-\frac{d^3s}{dx^3}$ must be positive.

$$-\frac{d^3s}{dx^3} = -2K_2 S_\infty + 6K_2 x,$$

therefore

$$x = \frac{1}{3} S_{\infty},$$

which corresponds to a minimum since

$$-\frac{d^2s}{dtdx^2} = 6K_3.$$

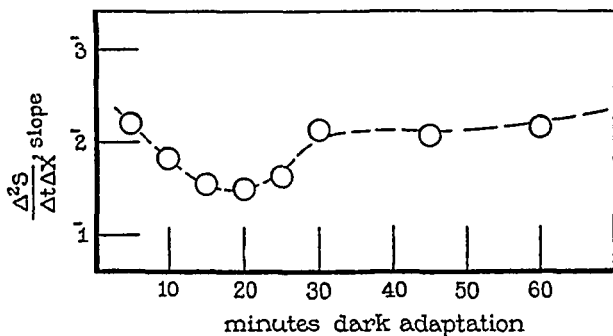


FIG. 7. The second order "dark" formation of S requires that the rate of light adaptation (i.e., change of $-\frac{ds}{dt}$) should be a *minimum* when $\frac{ds}{dt}$ is increasing most rapidly. The minimum found shows precise agreement with this requirement.

The fuller formulation is

$$\begin{aligned} -\frac{ds}{dt} &= K_1 (S_{\infty} - x) - [K_2 + K_3(S_{\infty} - x)] x^2 \\ &= K_1 S_{\infty} - K_1 x - (K_2 + K_3 S_{\infty}) x^2 + K_3 x^3; \\ \frac{d^2s}{dtdx} &= -K_1 - 2(K_2 + K_3 S_{\infty})x + 3K_3 x^2, \\ \frac{d^3s}{dtdx^2} &= -2(K_2 + K_3 S_{\infty}) + 6K_3 x; \end{aligned}$$

at inflection,

$$6K_3x = 2(K_2 + K_3S_\infty)$$

$$x = \frac{1}{3} \left(\frac{K_2 + K_3S_\infty}{K_3} \right) = \frac{1}{3} \left(\frac{K_2}{K_3} + S_\infty \right)$$

therefore

$$x = \text{a little} > \frac{1}{3} S_\infty.$$

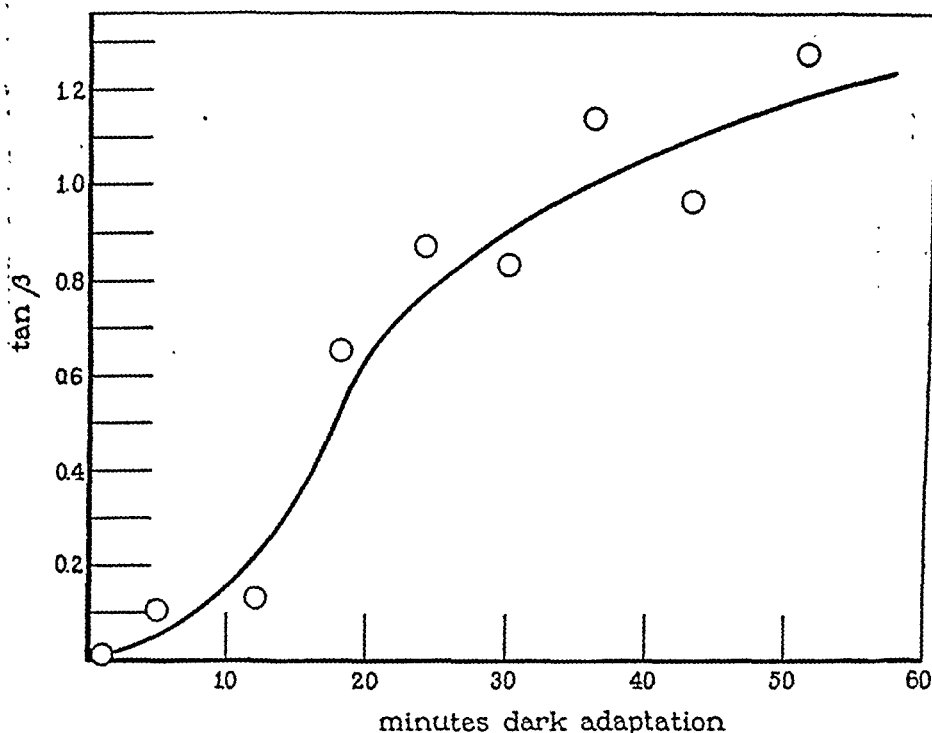


FIG. 8. A curve of dark adaptation as derived from single measurements of β with a number of slugs during the first minute of exposure to light subsequent to various periods in darkness following practically complete light adaptation. In this case, β corresponds to the values at 0.5 minute in Table I. Theory requires that the inflection of this graph should occur relatively earlier than in Fig. 2, which is the fact.

Hence the *rate of light adaptation*, with the test light of constant intensity, must pass through a minimum when x (i.e., $S_\infty - S_t$,

or $S_{\infty} \frac{(R - 1)}{R}$, has been reduced to slightly greater than $\frac{1}{3} S_{\infty}$.

The minimum found (Fig. 7) is at $t = 20$ minutes or a little less; this corresponds precisely to the region of the inflection point in the previous curve (Fig. 6) describing formation of S in the dark, and indicates that the rate of light adaptation is least when the calculated

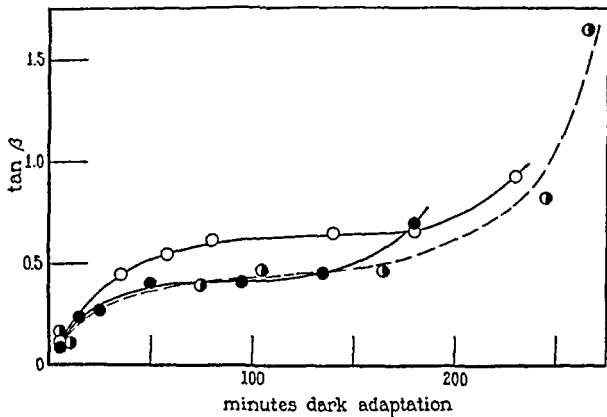


FIG. 9. Curves of dark adaptation as derived from repeated tests with the same individuals, at intervals during their photic adjustment in the dark. The shape of these graphs is to be contrasted with that in Fig. 8. The explanation is given in Fig. 10. (These curves were obtained from experiments at a temperature of about 15° , hence the time axis is extended by comparison with those earlier discussed.)

rate of formation of sensitive material is increasing most rapidly. The result is therefore quite reasonable.

Even if the tests for dark adaptation are made by the method of successive exposures of the same individuals to light during the progress of its dark adaptation, it is to be expected from the foregoing results that the change of the angle β should give a sigmoid curve when plotted against time. But since a certain amount of light

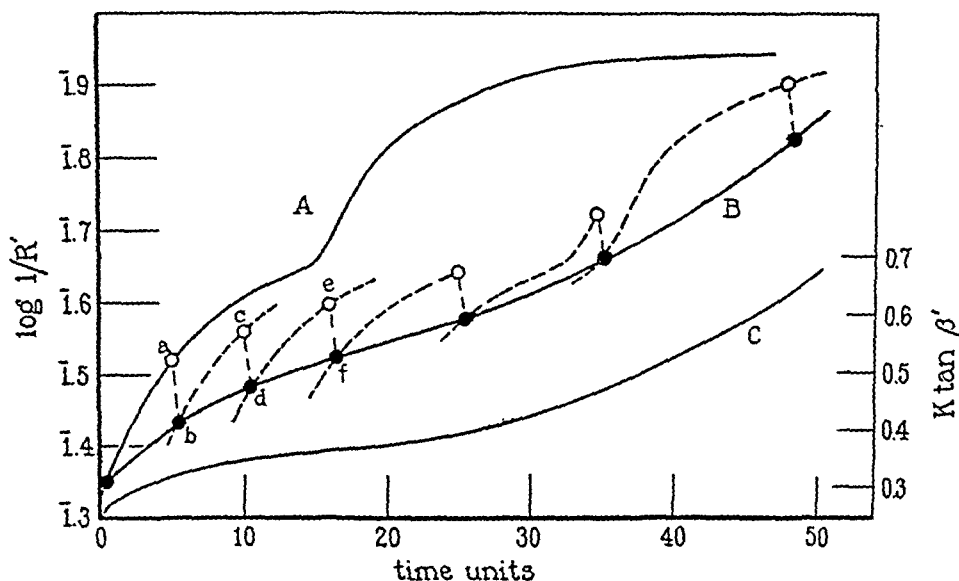


FIG. 10. The derivation of curves of dark adaptation of the two types obtained by the different procedures leading respectively to Fig. 8 and to Fig. 9. The fundamental curve of dark adaptation, that is of increasing initial excitability (β 0.5 minute in light), is given by the equations in the text or, graphically, by Fig. 6. Curve A is this graph. If taken at any point *a* on this curve and exposed to light, the excitability falls during the first minute of exposure to the level *b*, determined by the corresponding slope of the course of light adaptation at this level of adjustment (Fig. 1; Fig. 7). The magnitude of $1/R$ at point *b* may be gotten from such graphs as those in Fig. 8; or, in Fig. 1, from the ordinate intercepts at $t = 0.5$ minute (if the temperature and the intensity of the test light correspond; here, we are dealing only with the general *shape* of the resulting graphs). The curve of $1/R'$ is computed precisely in the manner of that for $1/R$, but on the basis of the ordinate intercepts (Fig. 1) at 0.5 minute exposure. On return to darkness, $1/R'$ begins again to increase, but now starts from level *b*. At intervals, this is all repeated. The continuous history deduced for $1/R'$ is then given by the *dotted line*. The observed change of excitability with time since first being placed in darkness should have the form given by the *heavy line*, which agrees with that found experimentally (Fig. 9); in the latter case the time axis is extended because the temperature was lower. The exact shape of curve B, particularly at the two ends, will depend on the frequency with which tests are made. There is thus a considerable interval over which the excitability does not change very much, when repetitive stimulation is employed. Curve C is curve B transformed to the same coordinates as Fig. 9.

adaptation is undergone during each interval of exposure, nothing can be gotten directly from such a curve (Fig. 9) whereby to test the quantitative formulation of the process. But it can be foretold that the shape of the curve under these conditions ought to be significantly different from that given in Fig. 7. Several curves obtained by taking single readings of β with slugs dark-adapted for different intervals are given in Fig. 8. The direct synthesis of such curves is explained in Fig. 10, without resort to the formulæ, and the result can fairly be taken as a striking justification of the form of interpretation here advanced.

VII.

The equation for the course of light adaptation as already given (10) may be written (again neglecting the suppressed velocity constant K'),

$$-\frac{ds}{dt} = 2K_2S_\infty S_t^2 - K_2S_t^2 + (K_1 - K_2S_\infty^2)S_t, \quad (12)$$

or

$$-t = \int \frac{ds}{2K_2S_\infty S_t^2 - K_2S_t^2 + (K_1 - K_2S_\infty^2)S_t},$$

whence

$$\begin{aligned} -t = \frac{1}{K_1 - K_2S_\infty^2} & \left\{ \frac{1}{2} \log \frac{S_t^2}{(K_1 - K_2S_\infty^2) + 2K_2S_\infty S_t - K_2S_t^2} \right. \\ & \left. + \frac{K_2S_\infty}{\sqrt{K_1K_2}} \tanh^{-1} \frac{K_2(S_\infty - S_t)}{\sqrt{K_1K_2}} \right\} + C, \end{aligned}$$

where C is the constant of integration. In this case, $S_t = S_\infty$ when $t = 0$;

therefore

$$\begin{aligned} -t = \frac{1}{K_1 - K_2S_\infty^2} & \left\{ \frac{1}{2} \log \frac{K_1S_t^2}{S_\infty^2(K_1 - K_2S_\infty^2) + 2K_2S_\infty S_t - 2K_2S_t^2} \right. \\ & \left. + \frac{K_2S_\infty}{\sqrt{K_1K_2}} \tanh^{-1} \frac{K_2(S_\infty - S_t)}{\sqrt{K_1K_2}} \right\} \quad (13) \end{aligned}$$

At early stages of light adaptation, such as are measured in the curves in Fig. 1, beginning at any level of dark adaptation ($S_\infty - S_i$) does not change greatly; and if K_2 is quite small, as it is now to be relative to K_1 , then

$$-t = K''' \log S + K''', \text{ very nearly.}$$

It will be noticed that this is the relationship already illustrated in Fig. 1, although the *linearity* of the curve relating $\log \tan \beta$ (\equiv , practically,² to $\log S$) to time did not enter into the nature of the data used in the subsequent deductions.³ Hence the conclusion now obtained from (11) is not to be regarded merely as "getting out something originally put into the formula."

VIII.

The present analysis of phototropic effects as influenced by adaptation is capable of being developed in several directions. We may mention, in addition to temperature relations, such points as: photic adaptation as affected by wave-length, and (possibly) polarization; dark adaptation subsequent to photic adaptation by light of particular wave-lengths as followed by means of recovery curves for excitation by light of other wave-lengths; and the changes of orientation when the geotropic vector is also varied. Experiments are being undertaken to explore these possibilities.

IX.

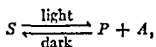
SUMMARY.

A method is described which measures the excitation of *Agriolimax* by light, during the progress of light adaptation, by assuming that the orientating effect of continuous excitation is expressed as a directly

² As $\log \left(-\frac{ds}{dt} \right) = \log [2K_2 S_\infty S_i^2 - K_2 S_i^3 + (K_1 - K_2 S_\infty^2) S_i]$; if K_2 is very small, this is nearly equivalent to $\log K_1 S_i$, for the same reason that the assumption of $R = \frac{S_\infty}{S_i}$ is very nearly sufficient (cf. §V).

³ Because the same *kind* of result is obtained if the analysis be based merely upon the values of β during the first minute of exposure to light.

proportionate tension difference in the orienting muscles of the two sides of the body. The tendency toward establishment of such a tension difference is caused to work against a similar geotropic effect at right angles to the phototropic one. This enables one to study the kinetics of light adaptation, and of dark adaptation as well. The situation in the receptors is adequately described by the paradigm



similar to that derived by Hecht for the differential sensitivity of various forms, but with the difference that the "dark" reaction is not only "bimolecular" but also autocatalysed by the reaction product *S*. The progress of dark adaptation is reflected (1) in the recovery of the *amplitude* of the orientation and (2) in the *rates* of light adaptation at different levels of the recovery; each independently supports these assumptions, for which the necessary equations have been provided. These equations also account for the relative variabilities of the angles of orientation, and, more significantly, for the two quite different kinds of curves of dark adaptation which are obtained in slightly different types of tests.

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GEOTROPIC ORIENTATION IN ARTHROPODS.

III. THE FIDDLER CRAB *UCA*.

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I.

Relations between extent of upward geotropic orientation on an inclined plane and the intensity of the effective gravitational pull have been studied and formulated in several different cases.¹ The formulations depend upon the view that the limitation of orientation is determined by the distribution of the pull of the animal's weight upon the muscles (of the body or of the appendages) of the two sides during progression. One test of this conception is obtained by examining the forms of the several equations proposed as descriptive for the mechanically different instances in which the morphology of the organisms, or their methods of progression, require the deduction of diverse analytical expressions. In general, it cannot be supposed that for animals differing fundamentally in this respect the same empirical rules will be found connecting the inclination of the surface and the extent of orientation. Since it is often desired to employ convenient empirical rules for purposes to which more rational equations do not easily lend themselves, it becomes to a certain extent important, as well as interesting, to supply in this way a qualitative check upon the "reality" of the differences between empirical rules for orientation as found with different organisms.

The orientation of such brachyurans as the fiddler crabs is particularly significant in this connection, since they creep sideways, and with the body not in contact with the creeping surface. The lateral

¹ For citations of earlier papers *cf.* Crozier (1928); Crozier and Pincus, (1926-27, a; 1927-28); Wolf (1926-27); Crozier and Stier (1927-28; 1928-29).

mode of progression in relation to heliotropic movements is already known (*cf.* Holmes, 1908; Loeb, 1912). Questions arising here, as to the inner, nervous mechanisms of orientation may perhaps be approached by the method of compounding geotropic and phototropic effects (*cf.* Crozier and Pincus, 1926-27, *b*; Crozier and Stier, 1928-29; Wolf and Crozier, 1927-28), particularly since it may be possible by certain treatments to force creeping to occur in a cephalad direction and thus at right angles to the direction of the more usual method. Nor should it be overlooked that with such crustaceans it may be possible to make experiments designed to test quantitatively the rôle of the statocyst organs in geotropism. Such inquiries require a preliminary study of the conduct of *Uca* upon an inclined surface.

II.

The extreme sensitivity of the fiddler crab to light made it necessary to work in a dark room under red light. The eyes were covered with a mixture of lamp black and collodion as an additional precaution, since the animals frequently respond to "safe" light. The creeping plane was of wood, about 1 meter square, over which bolting cloth was glued to provide a surface favorable for creeping.

The direction of progression is sideways and in a straight line. Starting from the center of a horizontal line drawn at the base of the creeping plane, the entire path of progression was observed, for a length of about 50 cm., its direction fitted by a straight edge, and the angle measured with a protractor. Creeping toward right or left seemed to be random, and no attempt was made to control it. Five readings to right and five to left were taken for each animal at each value of α , occasional creepings to one side or the other not being recorded in order to maintain equality of numbers. Otherwise, all trails were recorded except those in which creeping became interrupted or disorganized, usually because of light penetrating an imperfect eye-covering, excessive handling, or slipping. Such individuals were replaced in the aquarium and used again only after an interval of several hours.

The angle α was set at random to inclinations of between 15° and 70° , at multiples of 5° . Below 15° readings were hopelessly inconsistent and above $\alpha = 70^\circ$ the animals slipped. Table I gives the

values of θ at $\alpha = 20^\circ$ to 70° for one animal (No. 12, ♀) taken at random. The results with this individual are typical. The variation in θ is quite small. As with other female fiddlers tested, there is no bias of magnitudes of response to right or to left. The agreement between values of θ obtained from different individuals (♀♀) is

TABLE I.

Records of Angles of Upward Geotropic Orientation (θ) of Uca ♀ No. 12, at Different Slopes of the Surface (α).

α	20		25		30		35		40		45	
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right
	30.7	29.5	45.0	45.2	43.2	34.5	39.4	37.2	57.5	48.6	53.8	64.4
	30.8	20.5	27.0	50.9	48.7	45.7	36.5	54.6	59.2	62.1	59.2	64.0
	28.3	30.7	42.6	26.2	38.2	45.6	42.0	49.8	52.0	46.3	69.8	57.4
	16.5	21.5	32.5	41.4	35.8	45.3	54.0	44.0	55.2	49.6	68.5	65.7
	18.2	32.0	44.5	37.8	40.6	42.4	50.0	55.0	51.8	46.3	62.3	66.3
		17.7										
Av. θ	24.90	24.24	38.32	40.3	41.30	42.70	44.38	48.12	55.14	50.58	62.72	63.56
Mean	24.57		39.31		42.00		46.25		52.86		63.14	
α	50		55		60		65		70			
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right		
	59.8	62.7	75.4	69.9	79.5	76.3	82.5	79.6	80.5	83.2		
	69.8	65.4	69.8	79.9	73.6	78.2	76.6	80.4	84.1	81.0		
	60.2	63.8	74.6	73.5	79.5	81.4	82.5	83.7	82.3	79.5		
	65.5	70.4	75.7	72.1	74.8	78.7	77.8	81.7	79.0	83.6		
	65.2	59.1	68.8	75.7	74.7	76.5	76.7	79.5	78.5	80.0		
Av. θ	64.10	64.25	72.86	74.22	76.42	78.22	79.22	80.98	80.88	81.58		
Mean	64.19		73.54		77.32		80.10		81.23			

shown in Fig. 1. That effects of handling were successfully eliminated is shown by the fact (*cf.* Crozier and Stier, 1927-28; 1928-29) that when the creeping platform was tilted after the animal had begun to move upon it, values of θ were obtained agreeing precisely with those already recorded. Thus, with No. 9, tilting the surface to 30° gave a mean θ of 39.59° ; to 60° , $\theta = 76.47$ (*cf.* Table I).

III.

With female fiddlers the orientation angle θ is the same regardless of the right or left direction of the path. For the males, normally asymmetric as regards the position of the enlarged chela, this is not the case (Table II; Fig. 6). The means of *right* and *left* values of θ for

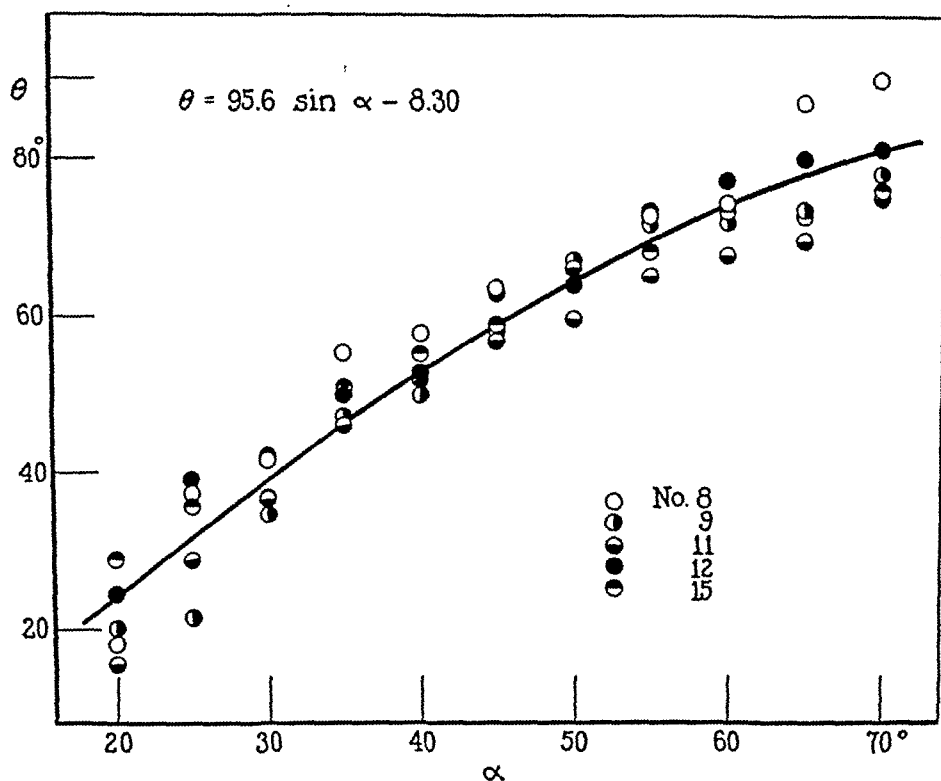


FIG. 1. Mean magnitudes of θ , the angle of the geotropically oriented path of progression, for female fiddler crabs on a surface with slope α° ; each point is the average of ten readings. The curve is transferred from that given in Fig. 2 as fitting the general averages.

the males, at each magnitude of α , agree very closely, however, with the corresponding mean θ 's obtained from the orientation paths of the females. The mean values of θ are collected in Table II.

The relation of θ to α is most simply expressed by a rectilinear connection of θ with $\sin \alpha$ (Fig. 2). A variety of other simple formulations fail to give an even approximately rectilinear plot. This case

is therefore similar to that of the beetle *Tetraopes* (Crozier and Stier, 1928-29), and with it differs sharply from various other instances¹ in which θ is a linear function of $\log \sin \alpha$.

In Fig. 2 the mean values of θ have been plotted for females, and for males and females, the averages being calculated as means of the average orientations to right and to left.

The variability of the measured θ 's does not adhere to the same rules as found with young mammals.¹ The differing mechanics of the

TABLE II.

Mean values of orientation angle θ at different inclinations (α) of the surface on which *Uca* creeps, for (1) ♀♀ Nos. 8, 9, 11, 12, 15, moving to *right* and to *left*, (2) ♂♂ moving to *right* and to *left*, with their averages; and (3) for ♂♂ Nos. 10, 14, 16 with large claw on the left side, (4) ♂♂ Nos. 13, 17 with large claw on the right.

α	(1) θ , ♀♀			(2) θ , ♂♂			(3) θ , ♂♂ Nos. 10, 14, 16		(4) θ , ♂♂ Nos. 13, 17	
	Left	Right	Average	Left	Right	Average	Left	Right	Left	Right
20°	23.17°	19.78°	21.48°	23.26°	18.18°	20.72°	21.37°	19.49°	25.14°	16.87°
25	32.94	32.14	32.58	32.97	31.78	32.38	33.31	36.57	32.63	26.99
30	38.77	39.14	38.96	38.73	40.09	39.41	38.71	45.61	38.75	34.56
35	50.33	50.12	50.23	45.34	44.92	45.14	45.01	49.65	45.62	41.19
40	54.26	53.15	53.70	49.71	50.32	50.02	47.73	52.06	51.68	48.58
45	60.87	59.83	60.34	56.88	58.22	57.55	53.83	59.04	59.92	57.40
50	65.09	64.39	64.75	63.53	64.32	63.76	61.27	65.61	65.79	63.03
55	71.55	69.20	70.38	72.27	70.14	71.21	68.20	68.53	76.34	71.75
60	73.82	72.29	73.06	74.48	73.86	74.17	69.21	73.22	79.74	74.50
65	76.93	76.67	77.26	78.39	79.33	79.22	71.76	76.43	85.01	82.22
70	81.06	79.23	80.15	84.56	82.48	83.62	81.77	82.59	87.35	82.37

two cases leads one to expect such differences, and the mean θ 's have not quite the same significance. To a reasonable approximation, $P. E. \theta$ here decreases linearly as $\sin \alpha$ increases (Fig. 3). Such relationships provide an "internal" check on the validity of the measurements.

IV.

In contrast to the mode of creeping in most forms previously investigated for geotropic orientation, the body of *Uca* is not directly

supported on the surface of the inclined plane. A primary condition for stability will therefore be that the vertical line from the center of gravity of the animal must pass through the plane within the area

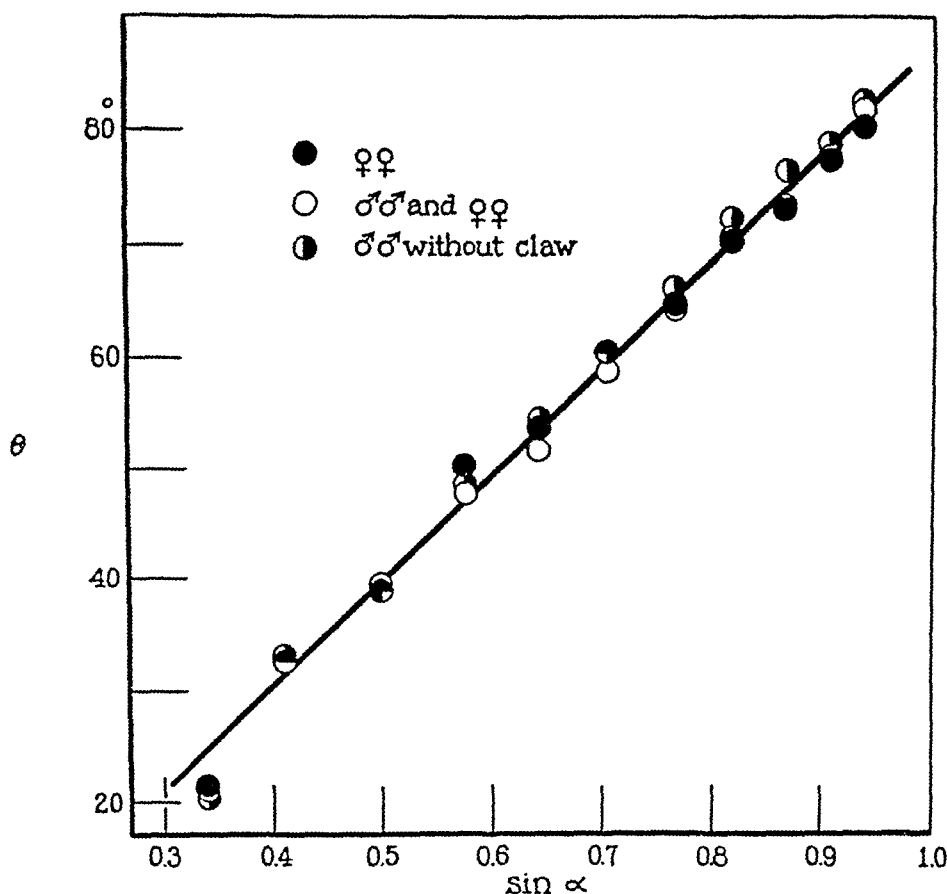


FIG. 2. Mean values of θ for ♀♀, and for ♀♀ and ♂♂, obtained as described in the text, give a rectilinear function with $\sin \alpha$. The same relationship holds for ♂♂ deprived of the large chela. The probable errors of the plotted points are less than 1° of arc.

delimited by lines connecting the points of support at the tips of the legs. An angle ρ may be defined as in Fig. 4, which is formed by the line connecting the tips of the most posterior legs with the intersection of creeping surface and the horizontal. Since direct observation shows

that the legs on the lower side of the crab are more widely extended, $\rho > \theta$, and we may write

$$\rho = \theta + m.$$

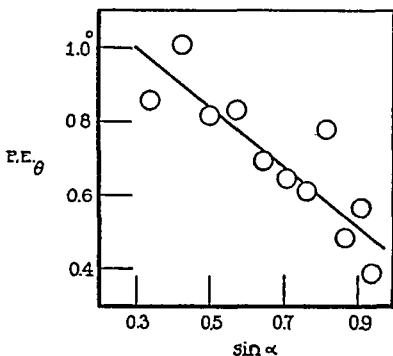


FIG. 3. The probable error of θ ($n = 50$) decreases in direct proportion to $\sin \alpha$.

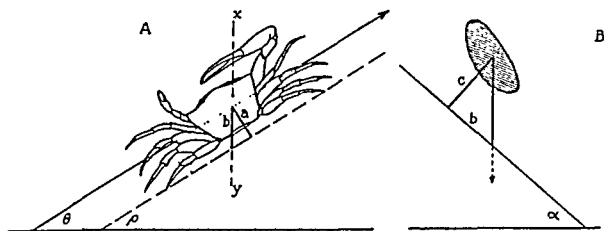


FIG. 4. *A*—Projection of *Uca* on the plane of creeping; direction of progression indicated by arrow. Diagrammatic.

B—Section through the vertical plane x — y . (Different scale.) See text.

If the height of the body above the surface, in a direction normal to

the surface, may be taken as constant, so that c in Fig. 4 is constant, then the minimum condition for stability may be obtained thus:

$$\tan \alpha = \frac{b}{c}$$

$$\cos \rho = \frac{a}{b},$$

hence $\cos \rho / \cot \alpha = \frac{a}{c} = \text{const.} = K$, if b and a may be taken as con-

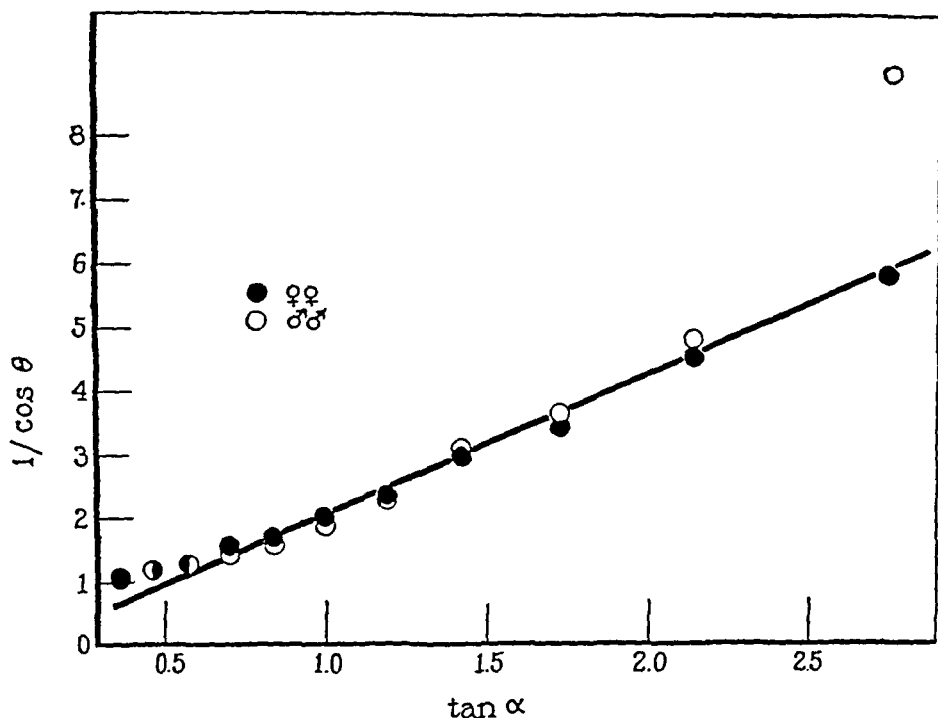


FIG. 5. The angle θ of upward geotropic orientation of *Uca* on a plane inclined at α° to the horizontal satisfied the relation $(\cos \theta) (\tan \alpha) = \text{const.}$; below $\alpha = 30^\circ$, the apparent magnitude of θ is a little too high. Solid circles, mean θ 's from measurements with ♀♀; open circles, ♂♂. The departure of the last point for the ♂ series may be due to slight slipping (2° of arc is enough to produce this departure). See text.

stant—i.e., if the relative spread of the legs is the same at all values of α . Since $\rho = \theta + m$,

$$\frac{\cos \theta \cos m - \sin \theta \sin m}{\cot \alpha} = K.$$

Taking data from the mean values of θ for ♀ ♀, the responses for these being symmetrical, by substitution in this equation and trial it is found that computed values of K tend to pass through a maximum (although the variation is not great), unless m be made quite small. With $m = 2^\circ$, K shows satisfactory constancy above $\alpha = 30^\circ$. In fact, m may be taken $= 0$, and $\cos \theta / \cot \alpha =$ sensibly *const.* above $\alpha = 30^\circ$. The discrepancy below $\alpha = 30^\circ$ might be presumed due to the fact that the orientation is not primarily controlled, at low values of α , by the endangering of the animal's stability, or else that

TABLE III.

Mean magnitudes of orientation angle θ at different inclinations (α) of the creeping plane, for *Uca* ♂♂ Nos. 14, 16, 17 before and after removal of large chelæ. Nos. 14, 16 bore large claw on left side, No. 17 on right. Trails have been averaged on basis of movement with side bearing large claw "up" (*i.e.*, ahead) or down.

α	θ With chela		θ Chela removed	
	Up	Down	Up	Down
20°	18.60°	21.18°	20.43°	19.18°
25	31.49	34.11	32.45	33.78
30	37.71	44.35	42.10	37.90
35	43.06	49.25	50.74	46.60
40	49.00	53.54	56.07	52.62
45	55.12	61.00	63.08	57.65
50	63.90	66.85	66.87	67.14
55	70.21	70.94	74.11	70.25
60	68.78	75.40	77.11	76.51
65	76.25	79.45	80.85	76.77
70	84.05	84.63	84.47	84.57

the measurements here are simply too variable because the crab occasionally turns too far. It is also probably the case that at low values of α c is greater than at higher slopes, because the legs are less widely extended; this would produce the deviation as found. The result of this treatment is adequately shown by the plot in Fig. 5. The departures at $\alpha = 20^\circ, 25^\circ$ are within the errors of the measurements.²

² It might be argued that the posture of *Uca* on an inclined surface is determined simply by its behavior as a moving object on a rough plane. It is difficult to make

Satisfaction of the elementary conditions for mechanical stability means that a certain class of tensions on the legs is released, namely those tensions required to prevent the crab from toppling over. Hence

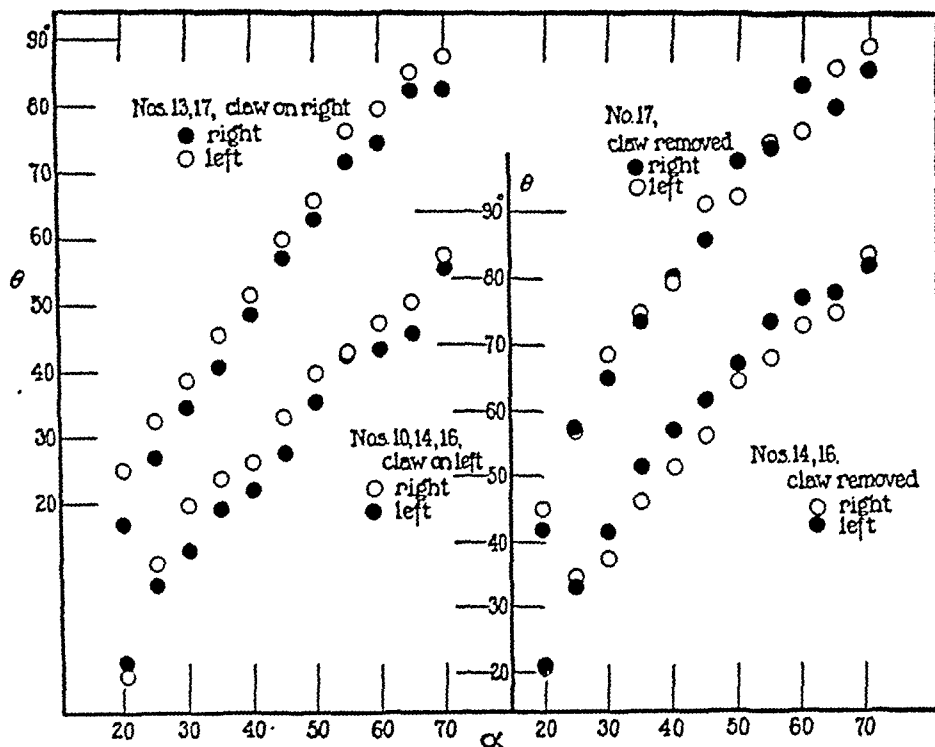


FIG. 6. Mean orientation angles to right and to left for 2 ♂♂ (13, 17) with large claw on the right side, and for 3 (10, 14, 16) with the large claw on the left, demonstrating lower θ 's when creeping is toward the side carrying the large chela. This relationship is obliterated when the large claw is removed.

the result here obtained is in no sense a contradiction of the "muscle tension theory" of the control of geotropic orientation, but is merely an extension of it to a somewhat more complex case.

refined application of this view, because of the nature of the crab's progression, but if we assume a "coefficient of friction" on the plane which acts normal to the axis of movement, then it can be shown that the condition for steady progression ought to be $\tan \theta / \tan \alpha = \text{const.}$ But this is not found. Moreover, this would leave unexplained the asymmetric response of normal males.

V.

The fact that the males of *Uca* characteristically bear one large and one small chela makes possible a further test of the muscle tension theory of geotropic orientation as applied to this case. Clearly, it would be expected, on the basis previously assumed, that consistently different values of the angle θ should result depending upon whether the large claw is carried in advance, that is "up," or "down." If the center of gravity is shifted by the presence of the large claw, then θ with the large claw "up" should be less than with this claw on the "down" side, regardless of whether the right or the left side bears the large claw. And the asymmetry of response should disappear if the claws are removed. In the case of female crabs no such difference should appear. The average θ , from trails to the right and to the left, taken together, should moreover be about the same for males as for females. These expectations are in fact all realized.

The records were obtained in such a way that "right" and "left" trails were measured separately with each animal and the results kept distinct. The mean values of θ for males with the large claw in the "up" posture and in the "down" (Table III) are plotted in Fig. 6. It is quite evident that θ is consistently lower if carried "up," regardless of the right or left location of the large claw. The reason for this, it is supposed, is that in the males the center of gravity is shifted to one side and anteriorly by the weight of the enlarged chela. The males used weighed from 2.600 to 2.973 gm., their chelæ from 0.697 to 0.795 gm. So large a proportion of the total mass being located in the fiddle, the center of gravity must be more anterior than in the females. Hence, to obtain stability, it is not necessary to turn through so large an angle θ when the claw is "up" as when it is on the "down" side (cf. Fig. 6), or in the absence of a large claw. (It has been pointed out previously that the mean of left and right θ 's is sensibly the same as with the females). If this be correct, the asymmetry evident in Fig. 6 should disappear with the removal of the claws of the males. Fig. 6 demonstrates that this expectation is in fact realized.

SUMMARY.

On an inclined surface the fiddler crab *Uca pugnax*, during sidewise progression, orients upward through an angle θ on the surface. The

extent of negatively geotropic orientation (θ) is a rectilinear function of $\sin \alpha$, where α is the inclination of the surface to the horizontal. This equation differs from that describing the geotropic orientation of various other animals. The difference is traced to the fact that from an initial position with the transverse axis of the body horizontal the crab is required to turn upward to an extent such that the vertical line from its center of gravity pierces the inclined surface within the base of support provided by the legs. This leads to the equation $\sec \theta / \tan \alpha = \text{const.}$, which is obeyed within the limits of precision of the measurements. This type of control of geotropic orientation represents an extension of the "muscle tension theory," and is in no sense in conflict with this view. The assumptions underlying the analytical expression connecting θ and α are verified by the asymmetry in the orientation of male fiddlers, which is shown to be due to the presence of the enlarged chela and which disappears when the claws are removed.

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SIGNIFICANCE OF THE CHEMICAL COMPOSITION OF THE SECRETING AND DRY MAMMARY GLAND TO MILK SECRETION.

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Of the constituents of milk, lactose is perhaps the most characteristic and least variable. A cow from the beginning to the end of her lactation or from lactation to lactation tends to produce a milk of relatively stable lactose content. Cows even of different breeds are much less divergent in the lactose percentages found in their milk than they are, for example, in the butter-fat percentages for the same milk. Lactose content has thus naturally become a starting point for the study of the mechanism of milk secretion. Gaines and Sanmann (1) have shown that the milk-secreting udder contains but negligible quantities of dextrose while large quantities of lactose are present. The amount of this lactose is closely related to the amount of milk which the udder is secreting at that particular time (1, 2) for if we determine the milk produced, its lactose per cent and total lactose, and compare this with the lactose content of the udder at a time corresponding to the normal milking period, the two lactose values closely agree as shown in Table I.

Table I shows that there is enough lactose present in the udder at the time of milking to more than account for the total amount of lactose produced by the cow in that milking. The average excess of lactose per cow is 0.0962 pounds. As the average lactose per cent of the milk drawn from these cows was 4.68, this difference represents an average equivalent of 2.1 pounds of milk remaining in the udder after the cow's udder is believed to be milked dry. The question naturally arises as to the justice of this comparison for it might be argued that the lactose represents a storage product to be drawn upon at some

subsequent lactation. That such is not the case is shown by the fact that the cow when dry, udder not secreting milk, has no reducing sugar in the udder tissue as shown in Table II.

The lactose of milk is believed to come from the dextrose of the blood (3, 4). Perhaps the best evidence for this conclusion comes from the analysis of the blood before entering the udder, generally

TABLE I.

*Total Lactose in Morning Milk Production and Total Lactose in the Udder at Time of Milk Production, Pounds.**

Cow No.	Lactose of milk production	Lactose in udder at time of milk production	Difference
	<i>lbs.</i>	<i>lbs.</i>	
137	0.129	0.206	0.077
97	0.187	0.353	0.166
114	0.194	0.315	0.121
221	0.333	0.323	-0.010
133	0.461	0.528	0.067
138	0.498	0.525	0.027
141	0.462	0.561	0.099
153	0.486	0.582	0.096
149	0.548	0.865	0.317
154	0.566	0.538	-0.028
111	0.596	0.718	0.122
1317A	0.639	0.784	0.145
148	0.649	0.614	-0.035
136	0.793	0.798	0.005
132	0.824	0.774	-0.050
124	0.856	0.931	0.075
144	0.876	1.090	0.214
1329A	1.035	1.288	0.253
1396A	1.227	1.313	0.086
5375A	1.264	1.441	0.177

* The customary unit of milk measure is the pound, 454 gm.

taken from the jugular vein, and blood leaving the udder, taken from the mammary vein. In the actively lactating udder the blood leaving the udder shows less dextrose than the simultaneously drawn blood of the general circulation. In the dry udder the blood of the general circulation shows no difference in its sugar content from that of the mammary vein. The general conclusion to be drawn from these facts,

i.e., that the lactose of milk is derived from the dextrose of the blood, is supported by the findings above as the udder in the lactating condition is shown to contain lactose whereas the dry udder does not.

TABLE II.

Lactose Found in Udder Tissue of Dry Cows.

Cow No.	Amount of lactose
118	None
155	"
167	"
170	"
44	"
61	"

TABLE III.

Chemical Content of Morning Milk and Udder with Contained Milk at Corresponding Times, Pounds.

Cow No.	Lactose		Fat		Ash		Nitrogen		Total solids	
	Milk	Udder	Milk	Udder	Milk	Udder	Milk	Udder	Milk	Udder
137	0.129	0.206	0.105	2.775	0.0230	0.1637	0.0218	0.2779	0.391	4.957
221	0.333	0.323	0.341	1.626	0.0494	0.1282	0.0414	0.2465	0.999	3.754
133	0.461	0.528	0.409	3.113	0.0684	0.1774	0.0532	0.3057	1.287	5.888
138	0.498	0.525	0.468	3.273	0.0905	0.2277	0.0649	0.3561	1.472	6.558
141	0.462	0.561	0.392	1.986	0.0890	0.6300	0.0720	0.4770	1.370	6.180
149	0.548	0.865	0.309	3.590	0.1060	0.3100	0.0690	0.4560	1.402	8.201
1317A	0.639	0.784	0.571	4.170	0.1060	0.6474	0.0802	0.5524	1.820	9.050
144	0.876	1.090	0.590	2.201	0.1530	0.3660	0.0930	0.5050	2.210	6.939
1329A	1.035	1.288	0.771	7.267	0.1624	0.5501	0.1299	0.9119	2.760	14.930
1396A	1.227	1.313	0.825	5.319	0.1628	0.4080	0.1137	0.6204	2.920	11.430
5375A	1.264	1.441	0.780	2.999	0.2018	0.4929	0.1264	0.7339	3.090	9.580

Chemical Composition of Milk as Related to the Chemical Composition of the Udder.

The chemical composition of the cow's udder when compared with the chemical composition of the milk produced throws some light on the raw materials stored by the udder for subsequent conversion into the constituents of milk. These data appear in Tables III and IV.

It is evident from an examination of Table III that at the time of milking there are sufficient materials in the udder to more than account for all the milk produced. This is particularly true of the fat, ash, and nitrogen. The lactose of the udder bears a much closer relation to the lactose of the milk than the other compounds of milk do to these same elements in the udder. This fact points to the conclusion that the lactose is formed from some raw product in the blood, probably dextrose, only as it is needed in the formation of milk. Fat, ash, and nitrogen have a fairly large excess reserve in the udder at all times. This is especially evident in the udder during the dry period as shown in Table IV.

TABLE IV.

Percentage Chemical Composition of the Udder during the Dry or Non-Lactating Period.

Cow No.	Lactose	Fat	Ash	Nitrogen	Total solids
44	None	17.37	0.79	2.28	34.8
61	"	18.42	0.73	2.39	34.2
118	"	40.49	0.59	1.64	52.1
155	"	29.87	0.78	1.67	44.8
167	"	72.08	0.23	0.93	78.5
170	"	60.91	0.34	1.16	69.5

The most noticeable changes in the composition of the dry udder are in the fat and the complete lack of lactose. The average composition of the udder tissue drained of milk is 13.3 per cent fat whereas the average composition of the dry udder tissue is 38.2 per cent. There is quite a wide variation in both groups, however, the composition of the drained udder tissue ranging from 7.6 to 20.7 per cent while that of the dry udder ranges from 18.4 to 72.1 per cent fat. The fat per cent of the secreting udder is significantly less than the dry udder even though this wide variation exists since for $n = 22$, $t = 5.2$ or P is much less than 0.01. These same relations also hold true for the water-free udder materials. The wide variation in both groups of data comes from the differences in the stages of lactation or gestation for the different cows. The fat of the dry udder is chemically different from butter-fat as the Reichert-Meissl number is practically 0 (0 to 0.4)

whereas that for butter-fat is larger than 24. The fat remaining in the udder after draining as much of the milk from the udder as possible approaches the Reichert-Meissl number of the dry udder. It seems unlikely that it would reach 0, however, as two cows which had the left side of the udder milked practically dry and then analyzed separately showed a Reichert-Meissl number of 11.8 and 14.4 respectively, whereas the right side of these udders containing 1 to 2 pounds more milk showed numbers of 11.1 and 13.0 respectively. The storage fat in the udder consequently appears different from butter-fat. The conversion of udder fat into butter-fat would therefore appear to progress somewhat faster than its utilization as in milk, a slight reserve being present.

Of reducing sugar the dry udder shows none whereas the udder in lactating condition contains its proportionate amount. The ash content of the dry udder shows a reduction to about half the amount found in the drained udder of the lactating cow (0.58 to 1.18 per cent). This difference is clearly significant as *P* is much less than 0.01. The nitrogen is practically the same in the dry udder as it is in the udder drained of milk, 1.66 to 1.79 per cent. The dry or quiescent mammary gland builds up a fat reserve of different Reichert-Meissl number than butter-fat, secretes no sugar, reduces its ash content, and has a nitrogen content practically that of the actively secreting udder.

SUMMARY.

The results herein presented furnish exact critical evidence for one more stage in milk secretion. Cows producing up to 30 pounds of milk at one milking are shown to have the lactose equivalent of all this milk in the udder when milking commences. The average excess of lactose found in the udder after subtracting the amount necessary for the contained milk is equal to 2.1 pounds. This represents the milk retained in the udder when the cow is believed to be dry. These conclusions are further supported by the fact that no sugar is found in the udder in the quiescent state.

The study of the total composition of the udder as fat, ash, nitrogen, and lactose, and of the contained milk shows that there is a large excess of fat, ash, and nitrogen in proportion to that necessary for milk formation. The excess of udder lactose over the milk lactose is much less.

The lactose would therefore appear to be formed from some element in the blood, probably dextrose, only as needed for the formation of milk.

The composition of the dry udder is quite different in certain respects from that of the actively secreting gland. It builds up a fat reserve of a quite different Reichert-Meissl number from that of butter-fat. It has no sugar, its ash content is reduced, and the nitrogen content is like that of the secreting gland.

Technique.—The method of extracting the lactose of Cows 97, 111, 114, 124, 132, 136, 148, 153, and 154 is described in (2). This method has been somewhat improved for the other cows indicated. The morning milk yield was determined as in the past. The cow was killed as before at the time when she had previously been milked for the morning milk yield. The udder and contained milk was then dissected off and separated into its two halves. The analysis was made separately on the two halves. The half to be analyzed was freed of any skin and teats, weighed and cut into inch cubes. The milk which drained out was caught and analyzed. The inch cubes were ground through a power meat grinder, passing through a plate carrying 1/16 inch holes. This material when well mixed was sampled and analyzed. Emphasis should be laid on the fact that this analysis should be made with as little delay as possible. The milk and drained milk from the udder were analyzed by the ordinary milk technique. The ground material was extracted for lactose, the lactose being determined with the polariscope. The material was dried to constant weight and extracted for the fat. The material was ashed in an electric oven. The nitrogen was determined by the Kjeldahl method.

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THE EFFECT OF CERTAIN ELECTROLYTES AND NON-ELECTROLYTES ON PERMEABILITY OF LIVING CELLS TO WATER.

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Although permeability to water must be regarded as one of the most fundamental properties of living cells, the degree of permeability may be surprisingly low (1), even temporarily nil (2) and varies with the physiological state of the cell (3). It is also thought to depend on the chemical composition of the medium, but direct evidence on this point is scanty. Experiments were therefore undertaken to show quantitatively whether cell permeability to water is regulated by electrolytes in the medium.

A satisfactory method for attacking this problem is to place the cell in anisotonic solutions, so causing water to enter or leave the cell under the driving force of osmotic pressure. The unfertilized egg of the sea urchin, *Arbacia punctulata*, is an excellent natural osmometer for this purpose; the amount of swelling or shrinking occurring in anisotonic solutions, and hence the volume of water entering or leaving the cell, can readily be measured under the microscope.

The effect of temperature and of the salt concentration of the medium on the rate of this process has been previously reported (4-6). In the present experiments, these factors have been held constant, and only the chemical composition of the medium varied. Under these conditions, change in rate of osmosis may be interpreted as change in permeability of the cell to water.

By permeability is understood the quantity of material (in this case, water) passing through unit area and unit thickness¹ of membrane in unit time under unit pressure. In order that results may be expressed

¹ We have assumed that the thickness of the membrane is constant.

in dimensions of permeability, the empirical equation² employed in previous papers has not been used here, although the velocity constant

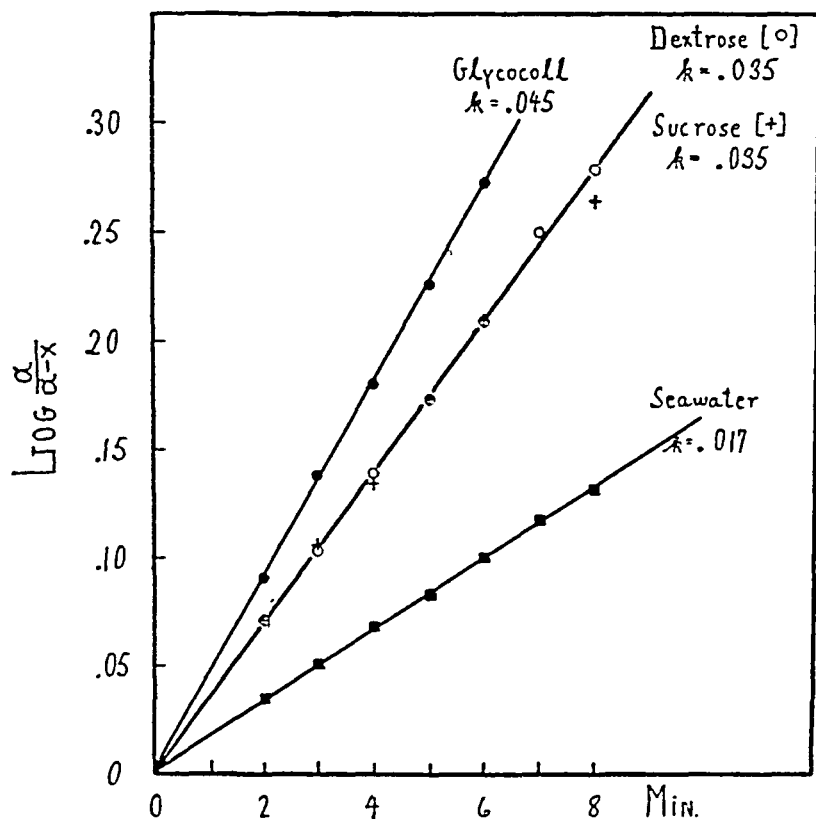


FIG. 1. The effect of non-electrolytes on rate of swelling in hypotonic solutions.

Data are taken from Table I. When $\log \frac{a}{a-x}$ (in which a is total increase in cell volume, and $a-x$ is volume increase up to time t) is plotted against times, the graph is a straight line, showing that the process follows the equation $kt = \ln \frac{a}{a-x}$, in which k is the velocity constant.

of that equation is approximately proportional to the permeability, provided that, as here, temperature and osmotic pressure of the

² $\frac{dx}{dt} = k(a-x)$, where a is the total volume of water that will cross the mem-

brane before equilibrium is established, x the amount that has already crossed at time t and k is the velocity constant.

medium are not varied. The empirical equation fits the present data, as shown in Fig. 1.

In this paper, results are calculated from the equation, permeability $= \frac{dV}{dt} / SP$ in which $\frac{dV}{dt}$ is cubic micra of water per minute, S is square micra of surface and P is atmospheres.

From the data in the accompanying tables, cell volumes were plotted against times, and a smooth curve drawn through the points. The rate of endosmosis was determined in each instance at the *second minute*, since earlier observations were frequently impossible on account of the time required for cells to settle. The rate at the second minute may be determined by drawing the tangent to the curve at this point, but since it is difficult to draw tangents accurately, it was found more satisfactory to determine the times required, beginning at the second minute, for the cells to increase 20,000 cubic micra in volume. The assumption made, that this portion of the curve is a straight line, while not strictly correct, introduces no serious error.

From the figure so obtained, increase in volume per minute was calculated. Practically identical rates were found by drawing tangents.

Surface and pressure were calculated for a point midway between the volume at 2 minutes and a volume greater by 20,000 cubic micra, i.e. volume at 2 minutes plus 10,000 cubic micra. Surface was calculated directly from the volume read from the curve at this point. Pressure inside the cell was calculated from the equation $P_t = P_o V_o / V_t$ in which P_o and P_t are osmotic pressures inside the cell at the first instant and at time t respectively, and V_o and V_t are the corresponding cell volumes. P_o was taken as 22 atmospheres, V_o was taken as the mean volume of 30 eggs of the same animal measured in sea water, V_t was read from the curve and P_t was calculated. The osmotic driving force is the difference between P_t and the pressure of the solution — 8.8 atmospheres for 40 per cent sea water. The values so obtained were substituted in the expression for permeability.

The technic of measuring volume changes in *Arbacia* eggs by means of a filar micrometer eyepiece has been previously described (4). In the experiments here reported, the cells were first washed in isotonic concentration of the solution to be employed, to remove electrolytes.

A drop of cell suspension was thoroughly mixed with about 20 cc. of this solution, and the cells were then allowed to settle. Temperature was maintained by a water jacket at $12 \pm 0.5^\circ\text{C}.$ ³

Effect of Non-Electrolytes.

The first step, preparatory to studying the effect of electrolytes, was to eliminate them from the medium and to learn whether the rate with

TABLE I.

The effect of non-electrolytes, contrasted with that of sea water, on the permeability of unfertilized *Arbacia* eggs to water. Solutions are isosmotic with 40 per cent sea water. Cell volumes are given in cubic micra $\times 10^2$. Each number represents the mean volume of 6 cells. In the bottom row is given the permeability, which is the number of cubic micra of water entering the cell per minute, per square micron of surface, per atmosphere of pressure. The temperature was $12^\circ\text{C}.$ (data are graphed in Fig. 1).

It is seen that permeability in non-electrolyte solutions is much higher than in sea water.

The mean volume of 30 control cells in 100 per cent sea water was 2060×10^2 .

Time	Glycocoll	Dextrose	Saccharose	Sea water
<i>min.</i>				
2	2475	2325	2410	2265
3	2710	2490	2595	2355
4	2900	2675	2725	2450
5	3075	2830	2900	2525
6	3250	2980	3050	2620
7		3145		2700
8		3245		2760
Permeability	0.142	0.097	0.103	0.050

which water entered the cells from hypotonic solutions was thereby affected. Accordingly cells were placed in hypotonic aqueous solutions of non-electrolytes—dextrose and saccharose, and also of glycocoll, which, on account of its slight electrolytic dissociation may be expected to behave toward cells like a non-electrolyte.⁴

³ Low temperature was necessary to prevent excessively rapid swelling in some of the solutions.

⁴ In most experiments solutions were made isotonic with 40 per cent sea water (sea water 40 parts, distilled water 60 parts). At Woods Hole this solution has,

The effect of these non-electrolytes is illustrated in Table I. It is seen that permeability to water is much greater in these solutions than in sea water of the same osmotic pressure (in most experiments it was about twice as great). Other experiments showed the differences in permeability in dextrose, saccharose and glycoll to be probably not significant.⁵

A possible objection to the significance of these results is that the hydrogen ion concentration was not the same in the several solutions, dextrose, saccharose and glycoll being on the acid side of neutrality, sea water on the alkaline. This objection is believed not to be valid, as preliminary experiments showed that increase in hydrogen ion concentration obtained by adding HCl to dextrose solution tends to decrease permeability.

Therefore, the rapid osmosis observed in non-electrolyte solutions is not due to increased hydrogen ion concentration, but either to the effect of non-electrolytes or to the absence of electrolytes.

Effect of Sodium and Potassium.

Supposing that the effect of non-electrolyte solutions were due to absence of electrolytes, it seemed possible that addition of electrolytes, especially of those occurring in sea water, to sugar solutions might lower permeability to that observed in sea water. All electrolytes might have this effect or only certain ones. Accordingly NaCl, which is the most abundant electrolyte in sea water, was added to hypotonic dextrose solution; the concentration of NaCl was 0.01 molar.⁶

according to Garrey (7), a freezing point of 0.73°C. Calculations based on data in the Landolt-Börnstein Tabellen show that 0.38 molar dextrose or 0.39 molar saccharose should be isotonic with this solution. 0.4 molar was assumed as approximately the isotonic concentration of glycoll.

⁵ Samples of dextrose from three manufacturers gave similar results. It is assumed that no significant penetration of these substances occurred during the short time that experiments lasted.

These non-electrolytes were selected because they are known not to penetrate cells readily. The choice of non-electrolytes is important because if substances were used which rapidly enter the cell, its osmotic pressure would increase and endosmosis would be accelerated.

⁶ Dextrose was chosen as the most convenient non-electrolyte with which to work.

Electrolytes were dissolved in distilled water in 1 molar concentration. the

The effect of NaCl and of KCl is illustrated in Table II. It is seen that the permeability values were even greater than in solutions of dextrose alone. Repeated experiments showed that there is probably no significant difference in the effect of Na and K, nor did these ions

TABLE II.

The effect of sodium, potassium, calcium and magnesium on permeability to water. The chloride of the several cations is added to dextrose in amounts sufficient to give 0.004 molar concentration of the electrolytes. Solutions are isosmotic with 40 per cent sea water. Each number represents the mean volume of 6 to 9 cells.

It is seen that in NaCl and KCl, permeability to water is of the same order of magnitude as in dextrose alone, while in CaCl_2 and MgCl_2 it is approximately the same as in sea water.

The mean volume of 30 control cells in 100 per cent sea water was 2035×10^3 .

Time	Dextrose	NaCl in dextrose	KCl in dextrose	CaCl_2 in dextrose	MgCl_2 in dextrose	Sea water
<i>min.</i>						
1						2210
1.5		2203				
2	2180	2310	2290	2245	2240	2285
2.5		2435				
3	2305	2560	2470	2365	2365	2370
3.5		2655				
4	2480		2580	2460	2435	2465
4.5		2900				
5	2655		2750	2535	2520	2545
6	2840		2855	2610	2595	2630
7	2955			2685	2635	2685
8	3070			2745		2740
Permeability	0.093	0.129	0.096	0.054	0.050	0.048

invariably increase the rate of osmosis above that occurring in solution of dextrose alone. Lower concentrations of NaCl and KCl produced similar though less definite results; higher concentrations led to rapid cytolysis.

amounts of these solutions added to dextrose being so small as not to alter its osmotic pressure significantly.

Effect of Calcium and Magnesium.

There remained the possibility that only certain of the electrolytes of sea water have a restraining action on osmosis. Since Na and K tended to increase permeability to water, it seemed possible that Ca and Mg would have the opposite effect. This proved to be the case. In the same Table (II), the value in hypotonic sea water is 0.048, in dextrose solution of the same osmotic pressure, 0.093. On adding CaCl_2 to this dextrose solution in amount sufficient to obtain 0.004 molar concentration, the permeability was reduced to 0.054, approximately the value in sea water. This result was regularly reproducible. A similar effect was produced by MgCl_2 .

Remarkably small amounts of Ca were found effective in slowing osmosis in dextrose, though a concentration of 0.00005 molar proved to be too low. Thus in one experiment in which the concentration of CaCl_2 was varied, the following results were obtained: 0.001 M, 0.044; 0.0005 M, 0.042; 0.0001 M, 0.049; 0.00005 M, 0.077.

These experiments indicate that the rapid osmosis observed in dextrose solutions is not due to the effect of non-electrolytes, but to absence of Ca and Mg. Also, the conclusion appears justified, that the relatively slow osmosis observed in sea water is due, at least in part, to the presence of these two bivalent cations.

It has been seen that the chlorides of Ca and Mg in dextrose solutions decrease permeability to the value found in sea water, whereas NaCl and KCl have no such effect. Numerous experiments invariably gave similar results.

Antagonism of NaCl and KCl with CaCl_2 and MgCl_2 .

As stated above, Na and K in dextrose solution did not always increase permeability more than did dextrose alone. In order to determine the effect of these cations more definitely the principle of salt antagonism was used. Having found a concentration of CaCl_2 in dextrose solution which was just sufficient to lower permeability to the value obtained in sea water, varying amounts of NaCl were added. In appropriate concentrations, NaCl antagonised the slowing effect of CaCl_2 , the degree of permeability being intermediate in value between that of Ca in dextrose and that in dextrose alone. K was just as effective as Na in this respect. A typical experiment is represented in

Table III. Ca could be replaced by Mg and similar antagonism with the univalent cations demonstrated. Such experiments show definitely that NaCl and KCl tend to increase permeability to water.

Preliminary experiments indicate that this method of studying salt antagonism can also be used quantitatively. Table IV shows that increasing amounts of NaCl added to CaCl_2 in dextrose give increasing values for permeability.

TABLE III.

Antagonism of sodium or potassium with calcium. In 0.0005 molar CaCl_2 in dextrose, permeability to water has the same low value as is usually obtained in isosmotic (40 per cent) sea water. Upon the further addition of NaCl or KCl (final concentration 0.01 molar), permeability increases to values intermediate between those in calcium-dextrose solution and in dextrose alone.

Each figure represents the mean volume of 5 to 7 cells. Volume of control in 100 per cent sea water, 2030×10^2 .

Time	Dextrose	KCl and CaCl_2 in dextrose	NaCl and CaCl_2 in dextrose	CaCl_2 in dextrose
<i>min.</i>				
1		2170	2015	2195
2	2285	2325	2145	2255
3	2470	2435	2295	2355
4	2595	2585	2405	2450
5	2735	2695	2520	2520
6	2890	2820	2645	2580
7	2995	2935	2770	2650
8		3045	2850	2720
Permeability	0.091	0.076	0.065	0.050

DISCUSSION.

These experiments indicate that the cations of sea water are important in regulating the permeability of the cell to water. There is an extensive literature on the effect of ions on permeability of cells and tissues to various substances.⁷ In most cases, as in our experiments

⁷ The literature on this subject is reviewed by Osterhout, W. J. V., Injury, recovery, and death in relation to conductivity and permeability, Philadelphia and London, 1922. Jacobs, M. H., in Cowdry, E. V., General cytology, Chicago, 1924. Hüber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 6th edition, 1926. von Tschermak, A., Allgemeine Physiologie. I, Berlin, 1924.

given above, Na and K have been found to increase permeability, Ca and Mg to decrease it. Particularly striking is the agreement between our results and those of Osterhout (8), who studied the effect of various electrolytes on the electrical conductivity of plant tissues, using this property as a measure of permeability. For these reasons it is probable that the action of ions stated above has general application.

TABLE IV.

Antagonism of sodium and calcium. Having determined permeability to water in solution of 0.0005 molar CaCl_2 in 0.38 molar dextrose, increasing amounts of NaCl are added to this solution. Permeability is seen to increase with the amount of NaCl added. Each figure represents the mean volume of 5 or 6 cells. The mean volume of 30 control cells in 100 per cent sea water was 2010×10^3 .

Time	CaCl_2 and 0.016 M NaCl	CaCl_2 and 0.008 M NaCl	CaCl_2 and 0.004 M NaCl	CaCl_2
min.				
1	2115			
1.5	2180			
2	2270	2245	2170	2230
3		2330	2265	2325
4	2525		2385	2420
5	2675	2520	2430	2480
6	2830	2640	2535	2545
7		2760	2675	2620
8		2890	2765	2710
Permeability	0.080	0.056	0.050	0.047

On the other hand the effect of non-electrolytes seems to vary with the material and method used (9).

The method employed by us permits direct measurement of the rate with which water crosses the cell membrane. The effect of different ions on permeability is striking and reproducible. Since the experiments were carried out at constant temperature and constant osmotic pressure of the medium, the conclusion is justified that the permeability of the cell to water is increased by NaCl and KCl, decreased by CaCl_2 and MgCl_2 .⁸

⁸ The mechanism of these changes in permeability to water has not been investigated in the present series of experiments. It may be said, however, that injury

SUMMARY.

1. Permeability to water in unfertilized eggs of the sea urchin, *Arbacia punctulata*, is found to be greater in hypotonic solutions of dextrose, saccharose and glycocoll than in sea water of the same osmotic pressure.

2. The addition to dextrose solution of small amounts of CaCl_2 or MgCl_2 restores the permeability approximately to the value obtained in sea water.

3. This effect of CaCl_2 and MgCl_2 is antagonized by the further addition of NaCl or KCl .

4. It is concluded that the NaCl and KCl tend to increase the permeability of the cell to water, CaCl_2 and MgCl_2 to decrease it.

5. The method here employed can be used for quantitative study of salt antagonism.

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to the cell is probably involved. Preliminary experiments of various types—such as fertilization tests on cells previously exposed for a few minutes to isotonic unbalanced solutions—suggest that NaCl and KCl are more toxic than CaCl_2 and MgCl_2 , and that recovery from injury may be possible to a certain degree. But on all these points more data are necessary before definite conclusions are drawn. It would be of considerable importance if it could be shown that under certain conditions, increase in permeability of the cell to water could be taken as a measure of injury.

OXIDATION-REDUCTION EQUILIBRIA IN BIOLOGICAL SYSTEMS.

I. REDUCTION POTENTIALS OF STERILE CULTURE BOUILLON.

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Oxidation-reduction reactions play an important rôle in the metabolism of all living cells. Since such reactions are often brought about in the medium outside of the living cell, a consideration of the oxidation-reduction equilibrium in sterile culture bouillon has seemed desirable because of its possible significance for the reductions manifested by bacteria in culture.

The only previous study of this problem was made by Theobald Smith,¹ who found that the reduction of methylene blue may be brought about by sterile sugar-free bouillon in the closed arm of the fermentation tube; in the presence of dextrose the reduction also of litmus takes place. These observations of Smith appear not to be generally known; they have not received the extension which they appear to warrant possibly because of the lack of suitable indicator substances and the want of theory covering their action as indicators of reduction.

Although the recent work of Clark² and his collaborators has met both of these deficiencies the electrometric measurement of oxidation-reduction equilibria provides a method which makes possible consideration of both the intensity and the capacity factors of reaction, and permits the statement of results in more precise terms than those of dye reduction. These considerations are peculiarly applicable to a poorly poised system such as culture bouillon, and experience in the

¹ Smith, T., *Cent. Bakt.*, 1. *Abt.*, 1896, xix, 181.

² Clark, W. M., *Pub. Health Reports*, 1926, suppl. 55, and earlier papers to which reference is given here.

preliminary work of the present investigation has shown the advantage of the electrometric method. Justification may be required for the use of this method in biological systems in which the essential reversibility of the reactions involved has not been established and in which the reacting substances have not been identified. The validity of the application is being established however by the work of recent investigators.²⁻⁶ The significance of electrode potentials for the state of reduction of a system has been discussed by Clark⁷ and Conant⁸ and need not be undertaken here.

Technic.

In the determination of reduction potentials in the present work attention has been limited, in order to simplify conditions, to bouillon prepared without the addition of carbohydrate. It was made in the usual way with meat-infusion. The pH was adjusted to 7.6 and stabilized by the addition of phosphate buffer to a final concentration of $M/15$, except in a few of the earlier experiments in which phosphate was not added. Sterilization was effected in an Arnold sterilizer, or in the autoclave at 10 lbs. for 10 minutes. In all of the later experiments the bouillon was autoclaved as described immediately before the experiment was started. The native dextrose of the medium was not fermented out.

The technic of measurement of the reduction potentials has been similar to that commonly employed with special attention to the purification of the metal used for the electrode. The electrode cell was either a weighing bottle, 4×5 cm. in dimensions, or a special vessel of similar size provided with a bottom outlet closed by a stop-cock. This was designed particularly for work to be reported on in a later paper. The mouth of the vessel was closed by a No. 9 rubber stopper, containing one central and eight peripheral perforations. These served for the introduction of (1) a separatory funnel through which solutions could be introduced into the cell, (2) the nitrogen inlet and outlet tubes, (3) the KCl-agar bridge tube, (4) a thermometer, and (5) four gold electrodes. All the glass tubes including that for the salt bridge were provided with stop-cocks which were lubricated with a mixture of paraffin and petrolatum. The agar bridge and the thermometer were sterilized separately and then fitted aseptically to the rest of the apparatus which had been assembled and autoclaved.

³ LaMer, V. K., and Baker, L. E., *J. Am. Chem. Soc.*, 1922, xlv, 1954.

⁴ Conant, J. B., and Fieser, L. F., *J. Biol. Chem.*, 1924-25, lxii, 595.

⁵ Dixon, M., and Quastel, J. H., *J. Chem. Soc.*, 1923, cxxiii, 2943.

⁶ Kenny, C. L., Dissertation, Columbia University, 1926.

LaMer, V. K., Kenny, C. L., and Sherman, H. C., to be published.

⁷ Clark, W. M., *Chem. Rev.*, 1925, ii, 127.

⁸ Conant, J. B., *Chem. Rev.*, 1926, iii, 1.

Nitrogen for deaeration was drawn from a commercial cylinder and allowed to pass through copper tubing filled with tightly rolled copper gauze. The only rubber tubing in the system was the short length which connected the copper tubing with the nitrogen inlet of the cell. The exposed surfaces of this rubber tube and of the rubber stopper were shellacked. The copper tubing with enclosed gauze was heated at its middle by an electrical coil or a large Bunsen flame and was cooled near the electrode cell by a water jacket. The nitrogen inlet tube of the cell was drawn out to a point with an internal diameter of 0.5 mm., and was placed so as to reach almost to the bottom of the cell, so that with the flow of nitrogen a stream of very small bubbles passed through the bouillon and assisted in keeping it agitated. No other stirring device was used, as necessity for it did not appear.

The preparation of the gold which was used as the electrode metal received the most careful attention. It was found early in the investigation that close agreement between different electrodes in the same solution could not be secured with the purest gold commercially obtainable. Consequently gold was purified first in solution and then as metal by cupellation with borax on a graphite cupel, in order to remove as far as possible traces of less noble metals and of silica. The solubility of silica in gold is worthy of remark. The purified gold button was fused to the purest platinum wire which could be obtained; this was sealed into glass tubing. Only those electrodes so prepared were used which were found equipotential when tested in ferri-ferro-cyanide solution and were equipotential or in very close agreement in bouillon exposed to air. I am indebted to Professor LaMer for this method of testing the electrodes.

A sensitive galvanometer was used as the null-point instrument. The advantage of an electrometer has not been apparent for this particular investigation. It has frequently been found necessary to render electrodes sensitive by slight polarization; any reversible change produced thereby in the system is reversed readily by alteration of polarity, and only such reversible reactions can be regarded as significant in the measurement of potentials. The potentials observed have been referred to the normal hydrogen electrode to give E_h values for pH 7.6 which was maintained in the bouillon throughout the series of experiments.

The reduction potentials of sterile culture bouillon are given in the form of time-potential curves. The initial potentials are variable and are poorly poised; in different experiments they have been found to lie between $+ 0.250$ and $+ 0.150$ volt. Individual electrodes during the first part of the curve may be in disagreement by as much as 50 millivolts, but have often been found to agree within 5 millivolts when the electrode metal has been purified in the manner described. Succeeding the irregular initial potentials is observed a steady negative drift or trend toward a region of greater reduction intensity.

The rate of this drift depends, with other factors, upon the rapidity with which oxygen is swept out of the system. If the exposed surfaces of rubber are shellacked, and if diffusion of oxygen through the cocks in the various tubes of the apparatus is prevented by careful fitting and lubrication, the oxygen is removed at the maximum rate. The potential then falls as shown for typical experiments with two different specimens of bouillon in Fig. 1, and within 5 or 6 hours

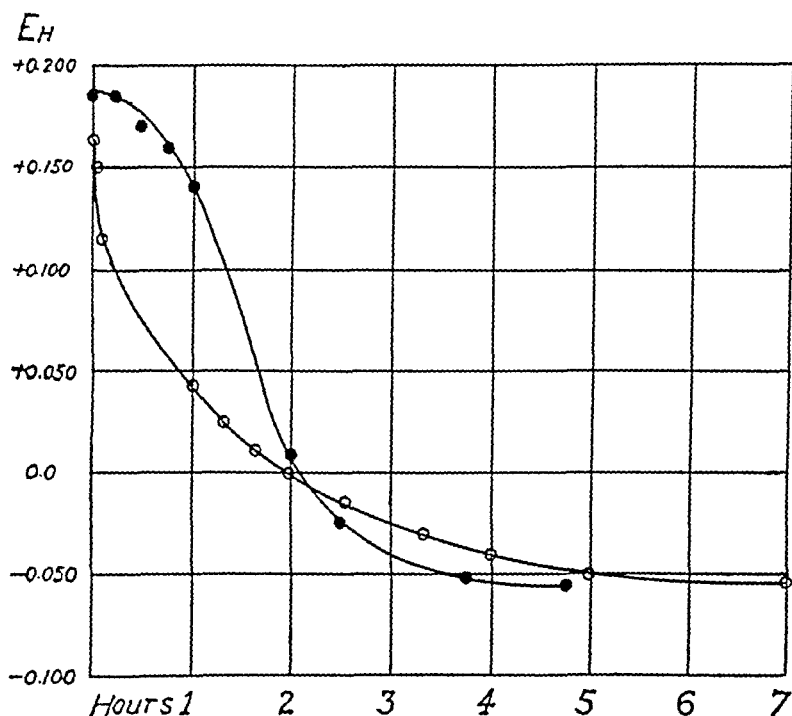


FIG. 1. Time potential curves of two specimens of sterile bouillon. Deaeration was started at the beginning of potential measurements and was continued throughout.

approaches a value between -0.050 and -0.060 volt. This appears to be a limiting value for the majority of lots of bouillon examined, and may be regarded as a characteristic potential for the system. After the technic had been fully developed and proper attention paid to exhaustion of oxygen from the nitrogen and to complete sealing of the apparatus against diffusion of oxygen from the outside, it was possible to duplicate results almost exactly with a

given lot of bouillon. The limiting value for the potentials and the time required to reach this have been very nearly the same for specimens of bouillon prepared at different times, when the same perfected technic was employed, although the configuration of the potential curve varies slightly.

Another observation has been made which is of considerable interest and is essentially a confirmation of the findings of Theobald Smith. In the earlier experiments in which exclusion of oxygen from the

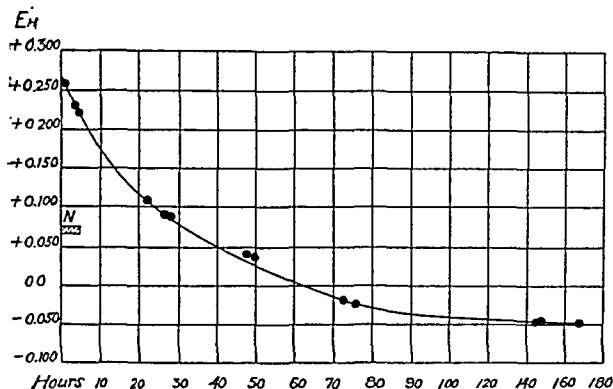


FIG. 2. Time potential curve of sterile bouillon, deaerated during the first 5 hours only of potential measurement.

system was not so complete as in the later ones, the potential did not reach the limiting value after 4 to 6 hours deaeration. If at this time the flow of nitrogen is discontinued and the vessel sealed against the entrance of oxygen by shellacking the rubber surfaces and covering stop-cocks with petrolatum, the potential continues to fall slowly and after 5 days or longer attains the limiting value above noted of -0.060 to -0.050 volt. The conditions are then similar to those studied by Theobald Smith: the oxygen in the bouillon within the closed arm of the fermentation tube becomes exhausted and the indicator dye is

reduced. Fig. 2 shows the course of potentials which corresponds to these events, although here no dye was present and a mechanism similar to that suggested by Kendall⁹ could not be involved.

If oxygen is admitted into the system after the potentials have begun to decline from the initial points or have reached the limiting value, the electrodes record an immediate shift to more positive values and may return to those initially observed if the aeration be sufficient. Renewed deaeration, or merely sealing of the apparatus against diffusion where this has occurred leads to a resumption of negative drift, which is at a more rapid rate if deaeration is carried out, and -0.060 volt is observed again as the limiting value of the potential.

DISCUSSION.

It has seemed worth while to consider the mechanism of the reduction processes of sterile bouillon, although the present state of knowledge does not permit much more than a suggestion of possibilities. The occurrence of life processes cannot play a part in the occurrences of the reduction described here. The technic of sterilization was such as to destroy all known forms of life, and cultures at the conclusion of each experiment included in the present consideration appeared sterile.

The combination of molecular oxygen with some constituent of bouillon, first observed by Theobald Smith,¹ recalls the respiratory function described by Hopkins¹⁰ for tissue residue. A small amount of a substance identical with or related to the water-insoluble muscle residue may be present in bouillon, contributing perhaps to its colloidal character. Bouillon contains glutathione extracted from chopped meat, and there may occur an action similar to that which Hopkins has described, but at a slow rate, in which glutathione acts as a catalyst in bringing about oxidation of the muscle residue by molecular oxygen. There is evident in any case the existence in bouillon of some autoxidizable substance the oxidation of which is accompanied by the reduction of electromotively active substances such as glutathione or indicator dye; the latter substances must be regarded as responsible

⁹ Kendall, E. C., *Science*, 1928, lxvii, 379.

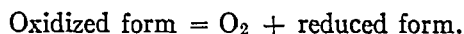
¹⁰ Hopkins, F. G., *Biochem. J.*, 1921, xv, 286.

for the potentials observed. The autoxidizable substance may undergo irreversible oxidation like many organic compounds, and would therefore be expected to give by itself no measurable oxidation potential.

It is significant that the limiting value of negative potential manifested by sterile bouillon of pH 7.6 should be so nearly the same as that observed by Dixon and Quastel⁵ in a solution of reduced glutathione and found by Clark² to be the equilibrium value in the reduction brought about by boiled yeast cells in the presence of glutathione. Glutathione is apparently an active constituent of bouillon. In this as in the reducing cell suspension studied by Clark,² reduction occurs as long as there is present a certain concentration of glutathione in the oxidized form, and does not proceed below the level of potential at which glutathione is completely reduced. The limited data suggest that in the catalytic oxidation of some autoxidizable substance by glutathione, the oxidized form of the latter is active, and the change from the disulfide to the sulfhydryl arrangement is the mechanism for electron transfer. This is only a development of the argument of Hopkins, but it emphasizes the idea that it is the glutathione which is primarily oxidized by molecular oxygen and that activation of the system consists in the formation of the oxidant of the electromotively active substance. This involves the conclusion that both the observed potentials and the oxidation of the autoxidizable substance measure the free energy of reduction of the glutathione or other electromotively active substance and implies that reduction does not proceed to a more negative level *because* there is no system present which could yield measurable potentials.

The capacity of bouillon to combine with oxygen as shown by the continued fall in potential after partial deaeration is not exhausted by standing for 9 months freely exposed to air in Erlenmeyer flasks, or as in Theobald Smith's experiments after several successive aerations and subsequent reductions of methylene blue. Consideration of other factors than those discussed above may therefore be required in the interpretation of the potentials observed, especially those noted while oxygen is being removed from the system by deaeration. As far as is known there are not present in bouillon electromotively active sub-

stances which stand in equilibrium with molecular oxygen according to the equation:



Conant⁴ found that oxyhemoglobin is not electromotively active; the potentials of the system methemoglobin-hemoglobin are affected by deaeration because of the removal of hemoglobin from participation in the electrochemical equilibrium by its transformation into the inactive oxyhemoglobin. Degradation products of hemoglobin may well be present in bouillon, and may provide a dissociable mechanism through which removal of oxygen affects the potentials.

We may question the existence of a suitable system to record more positive potentials than those observed, for although the addition of H_2O_2 in suitable amount raises the potential to a value exceeding +0.400 volt, there may be introduced thereby a new electromotively active system.

Since the removal of oxygen by deaeration or by combination with some constituent of the medium discloses a reduction intensity in bouillon corresponding to -0.060 volt, it is probable that any other process by which oxygen is withdrawn as reagent would result, in the absence of added factors of oxidation or reduction, in the attainment of the same potential level. Active bacterial respiration is such a process for the withdrawal of oxygen. But since *sterile* bouillon may attain the potential level indicated, the development of this degree of reduction intensity in bacterial cultures cannot be attributed to reductive processes directly dependent upon the activity of living cells. The potentials attained during growth of *B. typhosus* in sugar-free bouillon and their interpretation in the light of this conclusion will form the subject of a second paper.

The time potential curves of sterile bouillon, which show the course of potentials during deaeration with nitrogen are very similar to those obtained by Kenny⁶ in a study of the oxidation-reduction equilibrium of tomato and cabbage juice. The equilibrium value of tomato juice calculated for pH 7.6 corresponds closely with that observed for bouillon. It is probable therefore that the potential relations described for bouillon are not peculiar to that system, but involve factors which are concerned in the behavior of biological solutions in general.

SPECTROPHOTOMETRIC STUDIES OF PENETRATION.

IV. PENETRATION OF TRIMETHYL THIONIN INTO NITELLA AND VALONIA FROM METHYLENE BLUE.

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I.

INTRODUCTION.

Methylene blue has been generally regarded as a vital stain, but recent experiments^{1,2} have shown that it is not necessarily methylene blue that stains living cells. Using very large cells (*Valonia macrophysa*) yielding enough sap for spectrophotometric analysis, it was found that the dye in the sap of the vacuole is mostly, if not entirely, azure B, which is present as an impurity³ in the external solution of methylene blue. Methylene blue was not found in the sap even in reduced form.

In order to discover whether these results have general validity it became necessary to perform similar experiments on the fresh water plant *Nitella*⁴ and the marine alga *Valonia*; the results of this comparison are given in the present paper.

¹ Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, 425.

² Irwin, M., *J. Gen. Physiol.*, 1926-27, x, 927.

³ The following investigators have stated the presence of methylene azures in methylene blue. Scott, R. E., and French, R. W., *Milit. Surg.*, 1924, lv, 1. Conn, H. J., Biological stains, 1925, published by the Commission on Standardization of Biological Stains. Haynes, R., *Stain Technol.*, 1927, ii, 8. MacNeal, W. J., *J. Infect. Dis.*, 1925, xxxvi, 538. Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905, xiii, 358.

⁴ Preliminary reports have been published (cf. foot-notes 1 and 2, and Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927-28, xxv, 563).

II.

Methods.

1. *Conditions of the Experiments.*—The experiments were made with *Nitella flexilis* and *Valonia macrophysa*. The dye used for *Nitella* was dissolved in phosphate or borate buffer solutions at pH 5.5 or 9.2 (made up according to the standards given by Clark,⁵ and diluted ten times). The pH values of the buffer solutions were checked by means of the hydrogen electrode. The dye used for *Valonia* was dissolved in sea water at pH 9.5 (0.8 cc. of 0.2 N NaOH added to 100 cc. of Bermuda sea water). The pH value was determined as previously described.⁶ The concentration of dye in the sap was determined⁷ by matching the color of the tube containing the sap with that of the tube containing a known concentration of dye. The experiments were carried out at $25 \pm 0.5^\circ\text{C}$. Care was taken not to expose cells to direct sunlight. The solutions were changed every hour.

2. *Criteria for the Condition of the Cells.*—Irreversible injury is very often followed by collapse of the cell and disintegration of the chlorophyll bodies, but the loss of turgidity if not too far advanced is often found to be reversible. Reversible injury cannot be satisfactorily determined. Turgidity seems to be the best criterion, but the outcome will vary with the judgment and experience of the observer. If we employ this criterion and find that cells die in the solutions within 24 hours, are we justified in thinking that the cells are not injured when placed in such solutions for only 1 hour? Here again the outcome will vary with the personal judgment of the experimenter, though this is one of the methods used for controlling the experiments.

The least reliable method of detecting injury is to compare the mortality of the test cells (which have been transferred after the experiment from the dye solution to the normal medium) with the mortality of the control cells. If such cells begin to die very shortly after they are transferred we may conclude that in all probability severe irreversible injury occurred before the cells were removed from the dye solution, but if after a longer period they begin to die more rapidly than the control cells we are in no position to decide whether they were injured in the original solution or died as a result of the subsequent toxic action of the dye which had not been washed away (from the cell surface or the interior). In case these cells after transference are found to live just as well as the control cells, we are in no position to say that the cells had not been reversibly injured before transference since they may very well have been injured and recovered after transference.

⁵ Clark, W. M., The determination of hydrogen ions, Baltimore, 1920, pp. 81 and 83.

⁶ Cf. foot-note 2.

⁷ Test-tubes were employed for *Valonia* and capillary tubes for *Nitella*.

On the basis of the first two criteria the results presented in this paper involve no injury, unless otherwise stated.

3. *Method of Collecting the Sap for Spectrophotometric Analysis.*—Owing to the fact that the volume of sap collected from the vacuole of each cell of *Nitella* is very small there is danger of contamination of the sap from the stained cell wall at the time of extraction of the sap from the vacuole. Contamination may occur whether the sap is extracted by puncturing the cell wall with a capillary tube and drawing the sap into it or by cutting one end of the cell and allowing the sap to flow out into a glass container. To avoid contamination the cells were placed with only about $\frac{1}{4}$ of their length in the solution, as will be described later. About six cells were placed in each dish and were removed after a few minutes. It took about 1 hour to extract sufficient sap (1 cc.) for spectrophotometric analysis.

With *Valonia* the cells were completely immersed in the dye solution. After they were taken out of the solution, the superfluous dye was removed as much as possible in the following manner. The cells were wiped with cheese cloth dampened with the sea water in which they are normally kept, dipped in the sea water, wiped again with the dampened cloth, and finally wiped with a dry cloth. Even after all this washing and wiping, the dye still comes out onto the cloth when cells are wiped. The sap was extracted from the vacuole by puncturing the cell wall with a sharp capillary end of a glass tube into which the sap was drawn up. It took about 10 minutes to extract sufficient sap for analysis. Medium sized cells were chosen (the sap from each cell would have a volume of about 0.4 cc.). Unless otherwise stated the amount of dye penetrating from solutions at pH 9.5 into the vacuoles of such cells was sufficient to make the error resulting from contamination of the sap from cell wall at the time of extraction relatively negligible.⁸

The vial containing the extracted sap of *Nitella* or *Valonia* was shaken very gently several times to mix the contents. The sap was drawn up into a pipette and placed in the cell for immediate spectrophotometric analysis unless otherwise stated. The analysis itself took about $\frac{1}{2}$ hour. Measurements at critical wavelengths (between 645 μ to 670 μ) were determined twice, once before the regular series of measurements were made and a second time when the usual series were measured from 550 μ to 690 μ (proceeding from the lower to the higher wave-lengths). In no case were the readings found to differ which showed that the light had not affected the dye during the measurement.

4. *Accuracy of the Measurements.*—The concentration of the dye and the thickness of the layer of solution were adjusted as much as possible to ensure accurate measurements. The thickness of the layers employed for each curve is described in Table I.

The solvent for dye employed was (1) either the sap of *Nitella* containing about 0.11 M halides (mostly potassium chloride) at pH 5.5, or (2) diluted buffer solution (described in the text), or (3) sap of *Valonia* containing 0.6 M halides (mostly potassium chloride) at pH 5.8, or (4) sea water (0.58 M halides).

⁸ Cf. foot-note 2 (p. 929).

As already stated in a previous publication² the shape of the absorption curve differs with varying concentrations of dye and of salt present in the solvent. An increase in the concentration of either one tends to accentuate the secondary absorption maximum and obscure the primary, without disturbing actually the position of these maxima in relation to the wave-lengths. For example, irrespective of the concentration the primary absorption maximum of trimethyl thionin or azure B is at about 650 $m\mu$ while the secondary is at about 600 $m\mu$, though with an increase in concentration the primary absorption maximum tends to become less distinct, and the secondary more accentuated.

For identification of dyes the primary absorption maximum is more important than the secondary and if dilution of the dye accentuated the former, we might at first sight suppose that the greater the dilution the greater the accuracy *ad*

TABLE I.

Showing the Thickness of Layers of Solution in Centimeters Used for Spectrophotometric Analysis. Nature of the Solvents is Described in the Text.

Figures	Symbols	Thickness of layer
		<i>cm.</i>
1	All	1.0
2	×, ▲, and ● □ ○	1.0 0.6 0.2
3	▲ ●, ■, ×, and ○	1.0 0.3
4	All	1.0

infinitum. But this is not found to be the case. Beyond a certain dilution the measurement becomes very inaccurate on account of the distortion of the spectra.

The average eye is relatively insensitive in the spectral region of the absorption maxima of methylene blue and azure B or trimethyl thionin (between 645 and 670 $m\mu$), and particular care was taken to avoid the distortion of spectra which might have occurred through minor errors in measurements carried out on solutions which were too lightly or too strongly colored for optimum results. The collaboration of experienced technicians in the spectrophotometric analysis of dyes was obtained to insure reliable results.

5. *Acknowledgments.*—Unless otherwise stated, the measurements were made by the use of the Hilger wave-length spectrometer with a Nutting photometer or Bausch and Lomb (improved model) spectro-

photometer by W. C. Holmes⁹ of the Bureau of Chemistry, U. S. Department of Agriculture, Washington, D. C. I wish to express my deep appreciation to Mr. Holmes for his collaboration and to the Color Laboratory for its hospitality.

At the request of Mr. W. C. Holmes, a few measurements were repeated with a König-Martens spectrophotometer by Dr. K. S. Gibson of the Bureau of Standards, for whose collaboration as well as for the hospitality of the Bureau of Standards I wish to express my gratitude.

I also desire to thank the Marine Biological Laboratory at Woods Hole for the use of facilities during last summer.

I am greatly indebted to Mr. Holmes, Dr. B. Cohen, and to Sergeant French for samples of dyes. The samples are designated throughout the paper by the name of the donor placed in parentheses. The samples furnished by Dr. Cohen were Samples G and F of Dr. Mansfield Clark.¹⁰

III.

Spectrophotometric Analysis.

A. Experiments on Nitella flexilis.

(a) *Collected in New York.*—The experimental procedure was as follows: It was first of all necessary to determine whether the dye was stable in solution and to find out what penetrates the cell. This led to an investigation of errors due to contamination of the sap during extraction in proportion to the rate of penetration which in turn led to a study of the effects of injury. Consideration was then given to the important question whether azure B penetrates as such or is produced from methylene blue after the latter has entered the cell.

The results tend to indicate that azure B penetrates very much more rapidly than methylene blue.

⁹ Cooperating expert in the field of spectroscopy of dyes for International Critical Constants.

¹⁰ Clark, W. M., Cohen, B., and Gibbs, H. D., Hygienic Laboratory Bulletin No. 151, 1928, p. 174.

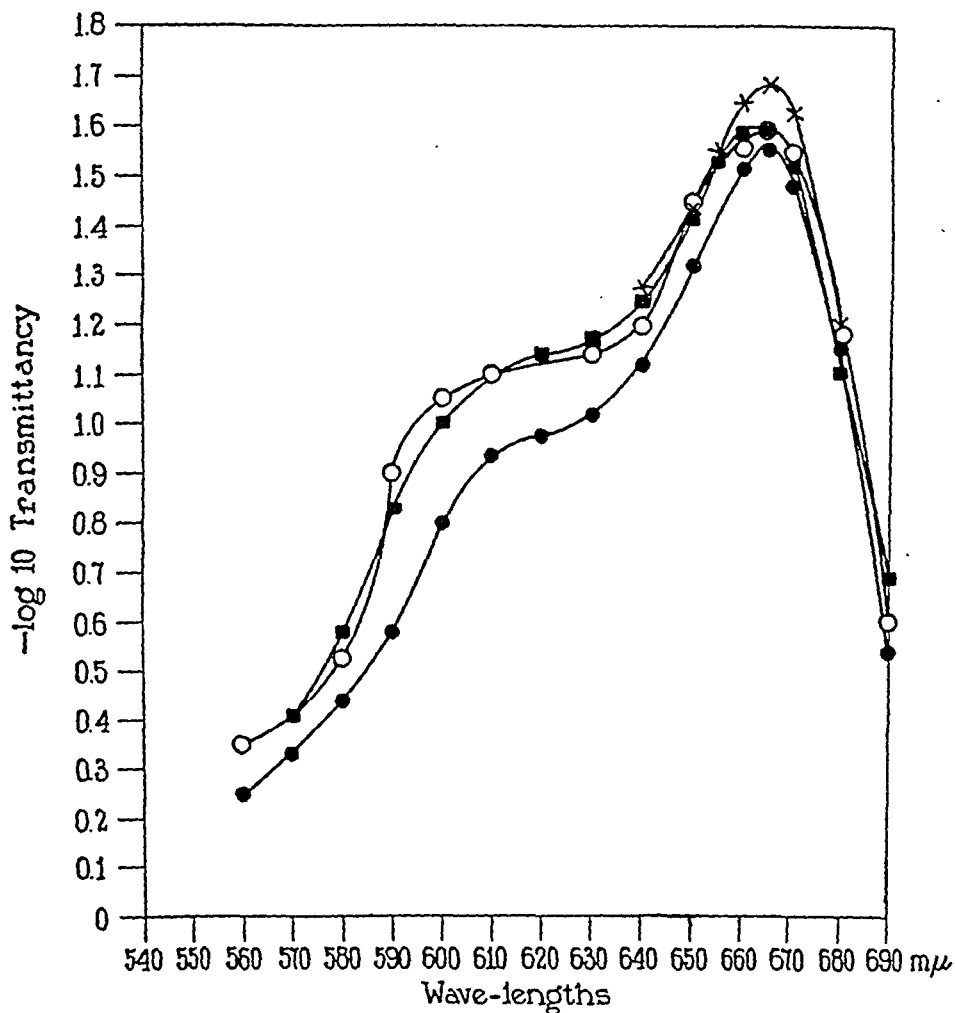


FIG. 1. Absorption curves showing that the sample of methylene blue in solution remains unchanged and that there may be contamination of the sap from cell wall at the time of extraction of sap from the vacuole of *Nitella flexilis* (New York) placed in methylene blue solution (French). Analysis made about 20 hours after extracting the sap or dissolving the sample. Symbol ● represents methylene blue dissolved in borate buffer solution at pH 9.2 or at pH 5.5. Symbol × represents the dye extracted by distilled water from the cell wall of uninjured cells stained in the methylene blue solution at pH 5.5 before the dye has penetrated into the vacuole. Symbol ■ represents the dye extracted from vacuoles of cells immersed completely in methylene blue solution at pH 9.2 where contamination may play a predominant rôle. Symbol ○ represents the dye extracted from vacuoles of cells placed in methylene blue solution at pH 5.5, where contamination occurred without any penetration. The heights of these curves have no significance other than attempts to select a condition as favorable for accurate measurement as possible under these circumstances.

1. *Stability of the dye solution:* Methylene blue solution (French) either at pH 9.2 or at pH 5.5 gives an absorption curve characteristic of methylene blue with absorption maximum at $664\text{ m}\mu$ (Fig. 1, symbol ●) whether it is examined immediately after dissolving or 3 hours later.

2. *Nature of the dye entering the vacuole when error from contamination was not avoided:* Analysis was made of the sap extracted from the cells which had been completely immersed for a few minutes in 0.01 per cent methylene blue solution (French) at pH 9.2. The dye in the sap gave an absorption curve characteristic of a mixture of methylene blue and azure B with a primary absorption maximum at about $661\text{ m}\mu$ (Fig. 1, symbol ■). Owing, however, to a severe contamination of the sap from the stained cell wall at the time of expressing the sap this result cannot be taken as conclusive evidence that the methylene blue present in the sap is a result of penetration rather than of contamination. Let us now consider the question of contamination.

3. *Contamination of the sap during the process of extraction:*

(a) In the solution just mentioned a very deep staining of the cell wall occurs before penetration of dye into the vacuole takes place. Dye extracted from the cell wall of such cells consisted chiefly of methylene blue with a primary absorption maximum at $664\text{ m}\mu$ (Fig. 1, symbol ×). This is important since the cell wall can give off this dye to the sap during the process of extraction.

Cells were placed in the 0.01 per cent methylene blue solution (French) at pH 5.5 or at pH 9.2 and were removed after the cell wall had become heavily stained but before any penetration into the vacuole took place. Such cells were removed from the dye solution, wiped, and were then placed in a small volume of distilled water until sufficient dye for analysis was extracted from the cell wall. Several extractions were analyzed and they were found to give the same results. Similar results were obtained when the extraction from the cell wall was made after the sap and the protoplasm had been removed.

(b) Sap known to owe most of its color to contamination at the time of extraction showed an absorption curve characteristic of methylene blue with an absorption maximum at $664\text{ m}\mu$ (Fig. 1, symbol ○).

Cells were placed in a solution of methylene blue (French) at pH 5.5 until the cell wall was deeply stained. The end of the cell was cut and a drop of sap was

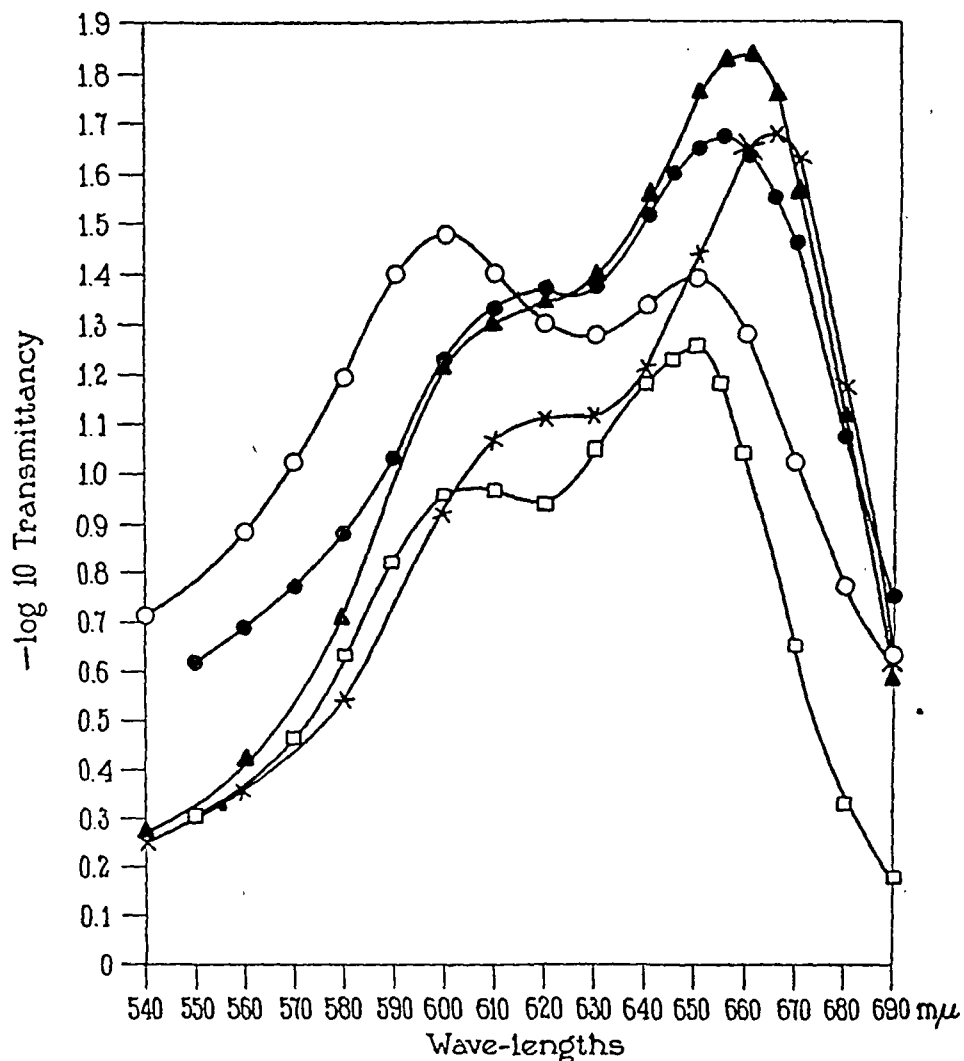


FIG. 2. Absorption curves showing the nature of the dye collected from the vacuole of *Nitella* (New York) placed in solutions of methylene blue (French) or azure B (Holmes), analysis being made about 20 hours after extracting or dissolving the sample. Symbol ● represents the dye collected from the vacuole of living cells after several minutes exposure to methylene blue solution at pH 9.2, where contamination was eliminated by allowing part of the cell to project out of the solution as described in the text. Symbol × represents methylene blue (French), at about the same concentration as the dye represented by symbol ●, dissolved in freshly extracted sap and analyzed after a period equal to the duration of penetration experiment and subsequent measurement. Symbol ▲ represents the dye collected in the vacuole of cells which have lost some turgidity as the result of injury during exposure to methylene blue solution at pH 9.2 where contamination was likewise eliminated. The dye in the sap was diluted with freshly extracted sap containing no dye since it was too concentrated for measurement. Symbol □ represents the pure sample of azure B (Holmes) dissolved in borate buffer solution at pH 9.2. Symbol ○ represents the dye collected from the vacuole of living cells after a few minutes exposure to the azure B solution at pH 9.2, where contamination was eliminated. The heights of these curves have no significance other than an attempt to adjust the concentration and thickness of the layer of solution so as to obtain as accurate a measurement as possible under these circumstances.

allowed to fall onto a glass slide. The drop of sap appeared colorless except for a very dark blue spot in the center which was caused by the dye diffusing from the cell wall contaminating the sap. The contamination occurred when the hanging drop of sap came in contact with the stained cell wall before it separated from the cut end and flowed out upon the surface of the glass slide.

These results show that the error may arise from contamination of the sap from the stained cell wall at the time of extraction. It was therefore necessary to avoid this contamination.

4. *Nature of the dye entering the vacuole of uninjured cells when contamination was eliminated:* To avoid contamination only about $\frac{3}{4}$ of the length of the cell was immersed in the methylene blue solution (French) and the remaining $\frac{1}{4}$ was allowed to rest on the edge of the dish. The latter was kept moist by wet absorbent cotton which, however, was separated from the dye solution by a short segment of the cell. Cells were thus placed in 0.01 per cent dye at pH 9.2 for a few minutes. The dye in the sap of such cells was found to give an absorption curve characteristic of chiefly azure B and a smaller percentage of methylene blue with a primary absorption maximum at $655\text{ m}\mu$ (Fig. 2, symbol \bullet). When the sap thus extracted was exposed to the air in an alkaline solution the color of the dye in the sap did not deepen, thus showing that there was no methylene blue in reduced form in the sap.

5. *Nature of the dye penetrating the vacuole of injured cells:* Since reversible injury is difficult to detect, we may inquire whether the azure B found in the vacuole as described under (4) is a demethylation product of methylene blue as associated with injury. If this were the case, the more injury the more azure B would be formed. To test this point experiments were carried out with cells which began to lose their turgidity during the experiment. It was found that methylene blue penetrated the vacuole more rapidly when cells began to lose their turgidity, which is shown by the fact that the dye from the vacuole then has a primary absorption maximum at $659\text{ m}\mu$ (Fig. 2, symbol \blacktriangle).

Obviously azure B penetrates much more rapidly than methylene blue unless injury has occurred. But it is possible that this penetration is only apparent and that after all it is methylene blue which penetrates and is transformed to azure B after it has entered. We must now consider this question.

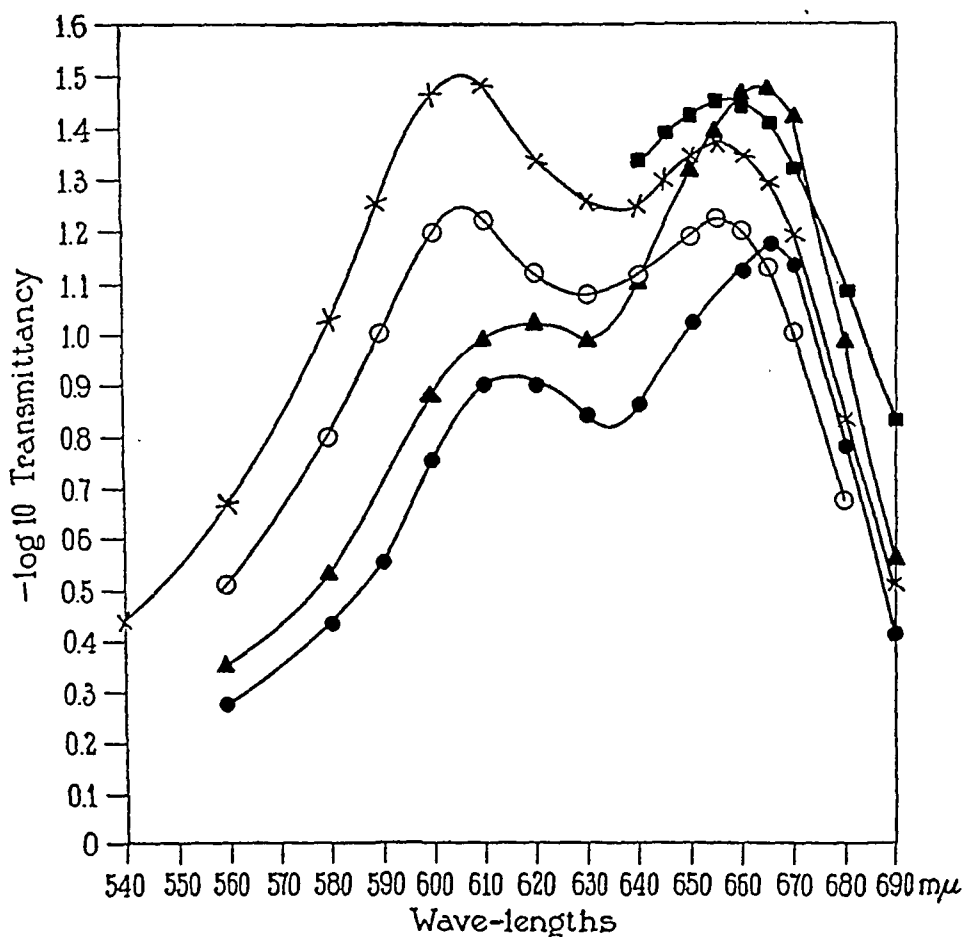


FIG. 3. Absorption curves showing the results obtained with *Nitella* (collected in Massachusetts) proving that the results are identical whether analysis is made immediately or 20 hours after extraction. Symbol ● represents the sample (Merck's medicinal) dissolved in freshly extracted sap and analyzed 4 hours later. Symbol ■ represents the dye collected from the vacuole of living cells placed in methylene blue (Merck's medicinal) at pH 9.2, when analysis was made immediately after extraction. Symbol ▲ represents methylene blue (Merck's medicinal) dissolved in buffer solution at pH 9.2 and analyzed 4 hours later. Symbol × represents the dye extracted from the vacuole of living cells placed in methylene blue (French) at pH 9.2, and analyzed immediately after extraction. Symbol ○ represents the dye extracted from the vacuole of living cells placed in methylene blue (French) at pH 9.2 and analyzed 20 hours after extraction. The heights of these curves have no significance other than an attempt to make measurement in the region where accuracy is possible under these circumstances. Contamination was avoided in all cases.

6. *Is the azure B in the sap produced from methylene blue which has entered the cell?* To throw some light upon this question methylene blue (French) was dissolved in freshly extracted sap (at about the same concentration as that found in the vacuole in penetration experiments) and allowed to stand for about the same length of time as was the case with the penetration experiment followed by the measurement. The dye in the sap was found to give an absorption curve characteristic of methylene blue with a primary absorption maximum at $664\text{ m}\mu$ (Fig. 2, symbol \times). This indicates that the sap is not able by itself to change methylene blue to azure B to any noticeable extent during the time of experiment.

(b) *Collected in Massachusetts.*—Owing to the fact that the extraction of sap from the vacuole of cells of *Nitella* required the cooperation of several technicians it was necessary in the experiments just described to extract the sap in New York City while the measurements were made by Mr. Holmes in Washington. Although these measurements were made about 20 hours after extraction of the sap, the validity of these analyses is shown by the fact that later, when it was possible to make in Woods Hole extractions followed immediately by measurements the dye in the sap (extracted from cells collected in Massachusetts, care being taken to avoid contamination) gave the same absorption curve as before whether it was measured immediately after extraction or 20 hours later. The primary absorption maximum was at $655\text{ m}\mu$ (Fig. 3, symbols \times and \circ). This primary absorption maximum closely resembles that obtained with New York *Nitella* (Fig. 2, symbol \bullet).

The same result was obtained when the experiments were repeated with methylene blue (Merck's medicinal) (Fig. 3, symbols \bullet , \blacksquare , and \blacktriangle).

Experiments with pure azure B. Although the sap has been shown to be incapable of demethylating methylene blue to azure B is the protoplasm able to do so? Since Holmes¹¹ has found that demethylation of azure B to azure A takes place more readily than demethylation of methylene blue to azure B, if we find azure B to penetrate the vacuole without demethylation, from pure azure B solution, we may be justi-

¹¹ Holmes, W. C., to be published later.

fied in concluding that it is unlikely that the protoplasm is capable of changing methylene blue to azure B. Dye from cells placed in azure B solution (Fig. 2, symbol \square) gives an absorption curve characteristic of azure B with a primary absorption maximum at $650\text{ m}\mu$ (Fig. 2, symbol \circ), thus showing that in all probability neither the protoplasm nor the sap is capable of demethylating azure B (contamination was avoided).

B. Experiments on Valonia macrophysa.

Dye from uninjured cells left for about $1\frac{1}{2}$ hours in methylene blue (French) at pH 9.5 gave an absorption curve characteristic of azure B with an absorption maximum at $650\text{ m}\mu$ (Fig. 4, symbol \bullet), while the external solution consisted chiefly of methylene blue with an absorption maximum at about $664\text{ m}\mu$ (Fig. 4, symbol \square).

If the conclusion is correct that the presence of azure B in the vacuole of cells placed in methylene blue solution is due to the more rapid penetration of azure B from the outside solution (which contains azure B as impurity) we should expect an increase in the rate of penetration on the addition of more azure B to methylene blue. This was found to be the case experimentally. Enough dye for analysis collected in half an hour in the vacuoles of cells (at pH 9.5) in 0.04 per cent methylene blue solution (Merck's medicinal) to which about 25 per cent of the same concentration of azure B was added, whereas during the same period not sufficient dye for analysis penetrated from the same sample of methylene blue solution to which no addition of azure B was made. Spectrophotometric analysis of the first solution showed a mixture of methylene blue and azure B with a primary absorption maximum at $660\text{ m}\mu$ (Fig. 4, symbol \circ) while the dye in the vacuole was chiefly azure B with a primary absorption maximum at $650\text{ m}\mu$ (Fig. 4, symbol \blacktriangle).

When cells are placed in pure azure B solution (Holmes) with a primary absorption maximum at $650\text{ m}\mu$ sufficient dye for analysis enters in a very few minutes and this dye is found to be azure B with a primary absorption maximum at $650\text{ m}\mu$ (Fig. 4, symbol \triangle) which indicates that neither the protoplasm nor the sap is capable of changing azure B.

All these measurements were made immediately after extraction of the sap. When some of the measurements in connection with *Valonia* were repeated by Dr. Gibson with a König-Martens spectrophotometer the results confirmed those previously obtained. Whether Samples F and G of Clark, or the sample of French is used, the dye which

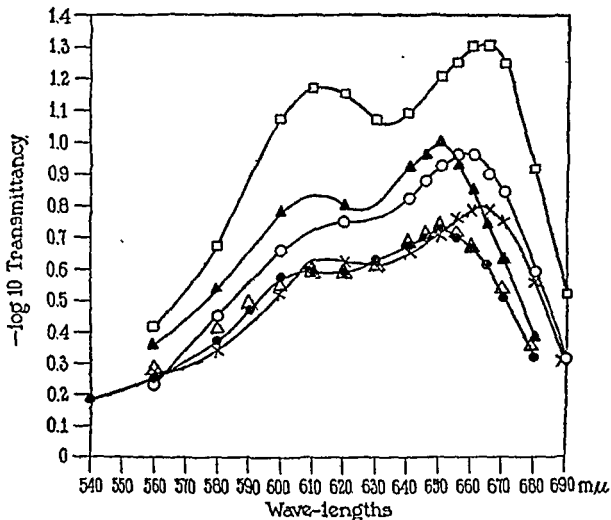


FIG. 4. Absorption curves showing the penetration of dye into *Valonia* from a mixture of azure B and methylene blue in comparison with the dye from methylene blue solution containing a trace of azure B as impurity. Symbol □ represents methylene blue (French) dissolved in sea water at pH 9.5 and analyzed 4 hours later. Symbol ● represents the dye which has penetrated from this solution in 1½ hours when analysis was made immediately after extraction. Symbol × represents methylene blue (French) dissolved in freshly extracted sap and analyzed 4 hours later. Symbol ○ represents a mixture of about 75 per cent of methylene blue and 25 per cent of azure B at pH 9.2. Symbol ▲ represents the dye which has penetrated from this solution in ½ hour when analysis was made immediately after extraction. Symbol △ represents the dye which has penetrated from pure azure B solution in less than ½ hour.

collects in the vacuoles of uninjured cells of *Valonia macrophysa* placed in methylene blue solution at pH 9.5 gives an absorption curve characteristic of dye chiefly consisting of azure B. With the sample of French, this absorption maximum is at 649 $m\mu$ while with Clark's samples it is at about 653 $m\mu$.¹² The absorption maximum of azure B is at 647 $m\mu$ and of methylene blue at 664 $m\mu$.

The difference in the absorption maximum between 650 $m\mu$ (from French's samples at pH 9.5) and 653 $m\mu$ (from Clark's samples at pH 9.5) is due to the fact that there is practically no methylene blue in the former while there is some in the latter. Methylene blue is detectable in the latter case because the rate of penetration of azure B is slower and the concentrations of dye found in the sap after 2 hours are much lower (at about 5.9×10^{-6} M and 7.9×10^{-6} M). It is difficult to determine whether this methylene blue in the sap is due to contamination of the sap from the stained cell wall at the time of extraction of the sap or to penetration. One way of determining this point would be to continue the experiments at pH 9.5 until the concentration of dye in the sap increased considerably and see if the presence of methylene blue would become masked by the increase of azure B in the sap. But this is inadequate on account of the danger of an added complication arising from possible injury.

The experiments previously² carried out with French's sample showed that at pH 9.5 with concentrations of dye up to about 1.6×10^{-5} M, unless the experiments were done with great care, a small amount of methylene blue was found with azure B in the sap giving an absorption maximum at about 653 $m\mu$. When there was more dye in the sap the methylene blue was masked by azure B, thereby giving an absorption maximum at 650 $m\mu$ which was identical with that of pure azure B. This indicates that at lower concentrations of dye in the sap there is a considerable possibility of error arising from contamination.

¹² Experiments were continued up to 3½ hours when one reading gave an absorption maximum at 653 $m\mu$ and the other at 656 $m\mu$. At such a slow penetration it is impossible to determine whether the presence of methylene blue (together with azure B) in the sap is due to very slight injury, contamination, or very slow penetration (much slower than the penetration of azure B). The results of such experiments must therefore be considered doubtful.

These results do not disagree with the theory presented in the text since they show that at higher external pH value the dye in the vacuole of uninjured cells consists chiefly of azure B while the external solution contains chiefly methylene blue with a trace of azure B as impurity. This indicates that azure B in form of free base possibly penetrates more rapidly than methylene blue.

IV.

DISCUSSION.

These results on *Valonia* and *Nitella* confirm those previously obtained with *Valonia*.^{1,2} The fact that by eliminating errors from contamination the primary absorption maximum in the case of dye penetrating from methylene blue solution at about pH 9.5 is at 650 m μ with *Valonia* while it is at 655 m μ with *Nitella* may be due to differences in the condition of the cells brought about during the experiments or to the normal compositions of the two types of cells or to the difference in the external media. Though there is this difference, the fact still remains that at about pH 9.5 both take up azure B from methylene blue solution containing a small amount of azure B as impurity. At this external pH value methylene blue is detected in the sap (1) when cells are injured or (2) when the contamination of the sap from the stained cell wall at the time of extraction occurs. The errors arising from these two sources are frequent so that experiments must be carried out with a great deal of care.

Has this azure B actually penetrated as such from the external solution or has methylene blue entered and become demethylated to form azure B? Holmes^{13,14} has shown that a progressive demethyla-

¹³ Since Holmes has found that demethylation of methylene blue to azure B at pH 11 does not occur instantly but only after standing for a long period this increase in the rate of penetration of azure B with a rise in the external pH value to pH 10.9 where at maximum the solution has stood for only 1 hour is not due to the increase in the concentration of azure B resulting from demethylation of methylene blue in presence of greater alkalinity.

¹⁴ Transformation of methylene blue to methylene azure in presence of alkalinity has been found by others. Bernthsen, A., *Ann. Chem.*, 1885, ccxxx, 137. Kehrmann, F., *Ber. chem. Ges.*, 1906, xxxix, 1403. Baudisch, O., and Unna, P. G., *Dermat. Woch.*, 1919, lxxviii, 4.

tion of methylene blue to azure B and then to azure A does not take place readily at a pH value below 10. Since the pH value of the sap is at about pH 5.8 and since the work of others¹⁵ indicates the pH value of the protoplasm in general to be considerably below 10, we may conclude that methylene blue cannot be demethylated by the protoplasm nor by the sap at their normal pH values unless they contain a substance capable of bringing about demethylation at a pH value below 10. Such substances must be absent from the sap for freshly extracted sap is incapable of demethylating methylene blue (at the concentration of the blue dye actually penetrating the vacuole) even during a period longer than that required for the penetration and its subsequent measurement in my experiments.

An additional reason for doubting that the protoplasm and the sap of these cells can demethylate methylene blue is that azure B is found to penetrate from pure azure B solution into the vacuole as such without undergoing demethylation to azure A. Since Holmes¹¹ has found that demethylation of methylene blue to azure B is brought about less readily than that of azure B to azure A, it is unlikely that the system which is incapable^{16,17} of changing azure B could transform methylene blue.

The azure B is not formed from methylene blue as a result of the change in the protoplasm or in the sap caused by an injury, because so long as cells are not injured azure B collects in the vacuole while as soon as it becomes injured methylene blue begins to penetrate freely.

The spectrophotometric measurement of the dye in the vacuolar sap of *Valonia macrophysa* has been recently repeated by M. M. Brooks,¹⁸ who concludes that the azure B present in the vacuole in my experiments is due to the transformation of methylene blue by the

¹⁵ A review of work on the pH values of the protoplasm is given by Chambers, R., *J. Gen. Physiol.*, 1926-27, x, 739.

¹⁶ This statement, however, must be made with reservation, since it has been found that in dogs the methyl group in position seven of the xanthine ring is most readily removed while in rabbits the opposite is the case (Krüger, M., and Schmidt, J., *Ber. chem. Ges.*, 1899, xxxii, 2677; *Z. physiol. Chem.*, 1902, xxxvi, 1).

¹⁷ When methylene blue is injected intravenously azure B is found in the urine and feces (Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905, xiii, 358).

¹⁸ Brooks, M. M., *University California Publications, Zoology*, 1927, xxxi, 90.

sap after extraction. She bases this conclusion on the assumption that my measurements were made after a lapse of time sufficient for the transportation of the extracted sap from New York to Washington. In view of the fact that my measurements, even when completed within 1 hour after extraction, gave absorption curves characteristic of azure B there is no basis for her conclusion.

If we suppose that azure B penetrates as such, diffusing through the protoplasm from an external solution containing mostly methylene blue, with only a small percentage of azure B, we may conclude that azure B penetrates the vacuole much more rapidly than methylene blue. This conclusion is supported by experiments showing that azure B collects in the vacuole much more rapidly from pure azure B than from methylene blue solution. The behavior of azure B is similar to that of many other basic dyes which are capable of forming free base and salt, since it is found to penetrate the vacuole much more readily as free base than as salt (the higher the pH value of the external solution the more rapid is the rate of penetration). This increases with rising pH value of the external solution for both azure B and methylene blue.^{19,20,21}

These results are in agreement with the theory²² that the rate of entrance of the dye is higher the more rapidly it enters the non-aqueous layers of the protoplasm and passes from them into the

¹⁹ The following investigators have found an increase in the rate of penetration of dye with an increase in the external pH value of methylene blue solution. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. MacArthur, J. W., *Am. J. Physiol.*, 1921, lvii, 350.

²⁰ My statement showing that the blue dye penetrated the vacuole of living cells from methylene blue solution more rapidly at pH 9.5 than at pH 5.5 (Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, foot-note 15, p. 572; 1926-27, x, 927) was contrary to the first conclusion drawn by Brooks, M. M. (*Proc. Soc. Exp. Biol. and Med.*, 1925-26, xxiii, 265; *Am. J. Physiol.*, 1926, lxxvi, 360), but my conclusion was confirmed by her in her later publication (Brooks, M. M., *University California Publications, Zoology*, 1927, xxxi, 90).

²¹ Baudisch and Unna have found that methylene azure enters chloroform and appears red (see foot-note 14). Kehrmann (14) has stated that methylene azure enters ether, chloroform, and benzene in form of a base and not in form of a salt.

²² Irwin, M., *J. Gen. Physiol.*, 1927-28, xi, 112; *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 127.

vacuolar sap. The entrance of the dye therefore depends on the concentration gradient and on the partition coefficient between the non-aqueous layers of the protoplasm and the aqueous phases.

In the present case the cell behaves as if the rate is controlled by only three phases (1) external solution, (2) one non-aqueous layer, and (3) aqueous sap in the vacuole. Since this will form the subject of the next paper it will not be discussed at length here.

On basis of this theory we may expect a very slow penetration of both azure B (in form of salt) and methylene blue, which might be detected if the dye penetrating the vacuole from methylene blue solution at pH 5.5 could be measured, but unfortunately the penetration is too slow for accurate analysis.

If methylene blue penetrates, its presence might be detected if there were little or no penetration of azure B. This might be ensured by placing cells in a sample of methylene blue exceptionally free from azure B but in this case the penetration of the blue dye is so slow that we are unable to obtain data and errors may result from injury or contamination.

Although the experiments show that unless cells are injured, azure B collects in the vacuole much more rapidly than methylene blue from methylene blue solution at a higher pH value, they do not tell us anything about the penetration of methylene blue into the protoplasm (which forms a layer so thin that no experiments suitable for our present purpose can be made on it directly). They merely point out the danger of drawing any conclusion as to the permeability of cells or the oxidation reduction potential of cells based on penetration of a blue dye from methylene blue solution without satisfactory determinations of the nature of the dye inside and outside the cell.

SUMMARY.

Spectrophotometric measurements show that it is chiefly the trimethyl thionin that is present in the sap extracted from the vacuoles of uninjured cells of *Nitella* or *Valonia* which have been placed in methylene blue solution at a little above pH 9. Whether these measurements were made immediately or several hours later the same results were obtained. Methylene blue is detected in the sap

(1) when the cells are injured or (2) when the contamination of the sap from the stained cell wall occurs at the time of extraction.

The sap is found to be incapable of demethylating methylene blue dissolved in it even on standing for several hours.

It is somewhat uncertain as to whether the trimethyl thionin penetrated as such from the external methylene blue solution which generally contains this dye as impurity (in too small concentration for detection by spectrophotometer but detectable by extraction with chloroform), or whether it has formed from methylene blue in the protoplasm. The evidences described in the text tend to favor the former explanation.

Theory is discussed on basis of more rapid penetration of trimethyl thionin (in form of free base) than of methylene blue, or of trimethyl thionin in form of salt.

THE DEATH WAVE IN NITELLA.

I. APPLICATIONS OF LIKE SOLUTIONS.

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Experiments with chloroform have led us to predict¹ that the current of injury will be positive when the cell is in contact with concentrated solutions (such as 0.1 M KCl) and negative with dilute solutions (such as 0.001 M KCl).

The experiments on cutting here described justify this prediction. They also reveal a new feature: the rapid spread of the effects of injury,¹ a study of which may assist our understanding of the propagation of stimuli. From the point where the cell is cut a wave of some sort, which we may for convenience call the death wave, passes along the cell, setting up at each point it touches a death process which has greater speed and intensity the nearer it is to the cut.

The experiments were performed on *Nitella flexilis*, arranged as shown in Figs. 1, 4, or 6: the changes in potential difference being recorded photographically by means of a string galvanometer. The technique has been fully described in a previous paper. The average temperature was about 23°C. but as the experiments were very brief there was little variation in any one experiment.

The results remained practically the same when the pH value of the solutions varied from 4 to 9.

Let us first consider the experiments in which sap² or 0.05 M KCl was applied at *A* and *B* (Fig. 1) after which the cell was cut at *Z* by means of a sharp knife insulated by rubber from contact with the hand (a clean cut was made by cutting downward against the paraffin

¹ This has been referred to in certain previous publications, cf. (a) Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, xi, 83; (b) Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 673.

² 0.05 M KCl gives the same results as sap for such experiments as are here described.

on which the cell rested). The cut caused *A* to become more positive after which the potential difference approached zero (Fig. 2).

Our interpretation of this phenomenon may be presented by describing how it arose. Our first experiments of this sort aroused the suspi-

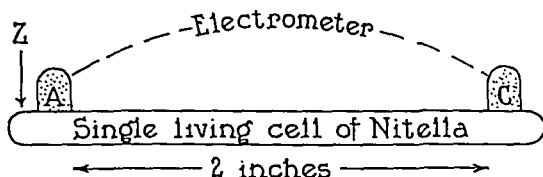


FIG. 1. Diagram to show the arrangement of the experiments (the cut is made at *Z*). Flowing junctions or pieces of cotton soaked in solution are applied at *A* and *C*. When necessary, with more concentrated solutions, cotton soaked in distilled water is applied for a short stretch between *A* and *C* but a space is left on each side of the cotton to prevent short-circuiting. In some cases the ends of the cells dip into cups holding solution.

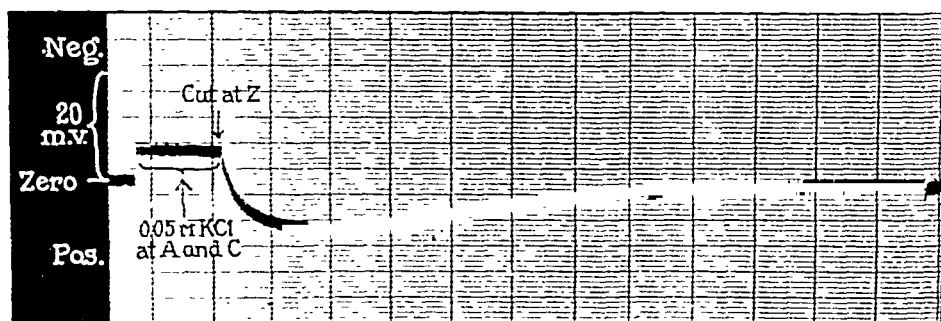


FIG. 2. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.05 M KCl at *A* and *C*. The curve (showing the potential difference of *A* with reference to *C*) is at first slightly negative but becomes positive when the cell is cut at *Z*, and then approaches zero. The vertical lines represent 5 second intervals. Selected as typical from 30 experiments.

cion that all the changes produced at *A* are followed by similar ones at *C*,³ and that the observed curve merely represents the resultant of the

³ The microscopic appearance of *C* changed in much the same way as that of *A* and so did its response to the tests for normality described in a former paper (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 417). The hypothetical curves of *A* and *B* imagined at that time were almost the same as those we use at present.

opposing potential differences at *A* and *C*. To illustrate this we made (Fig. 3*a*) a tracing of the curve in Fig. 2 and drew (Fig. 3*b*) hypothetical *A* and *C* curves to make it evident that the difference between them would give the curve in Fig. 2. But there was no way of testing

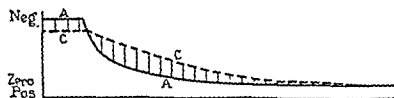
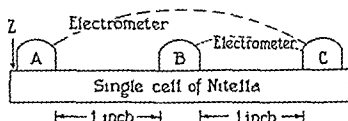
FIG. 3 *a*.FIG. 3 *b*.

FIG. 3 *a*. Tracing of the curve in Fig. 2 with certain ordinates drawn for comparison with Fig. 3 *b*.

FIG. 3 *b*. Theoretical interpretation of Fig. 3 *a*. Fig. 3 *a* shows only the observed potential difference but Fig. 3 *b* shows that this is equal to the difference between the hypothetical curve *A* (unbroken line) and the hypothetical curve *C* (broken line): each ordinate is equal to the one directly above it in Fig. 3 *a*. *A* and *C* are the (hypothetical) "true" curves of *A* and *C*. At the start *A* is more negative than *C* which accords with the fact that the curve in Fig. 3 *a* is negative at the start (this curve shows the potential difference of *A* with respect to *C*). When the value of *A* falls to that of *C* the curve in Fig. 3 *a* becomes zero; when *A* becomes positive to *C* the curve in Fig. 3 *a* becomes positive; the two curves then approach each other and the curve in Fig. 3 *b* approaches zero.

FIG. 4. Like Fig. 1 except for the addition of *B*.

this conception until it was observed that chloroform may kill *C* without immediately affecting *A* and *B*.¹⁵

It was found that when *C* is killed its electromotive force falls to zero and remains constant while the cut is being made at *Z* and

the resulting disturbances are recorded. This is illustrated by Fig. 5 in which the record starts after the disturbances⁴ produced by chloro-

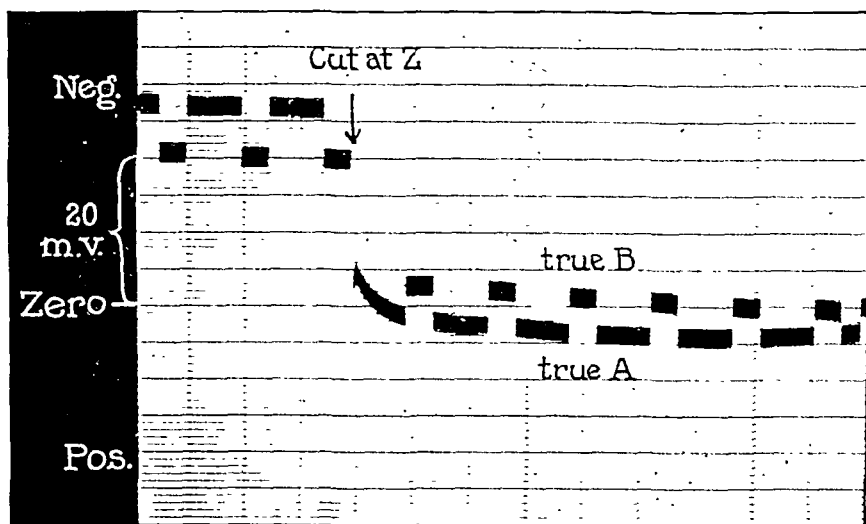


FIG. 5. Photographic record of potential differences, the experiment being arranged as in Fig. 4 with 0.05 M KCl at A, B, and C. The record begins after C has been killed by 0.05 M KCl saturated with chloroform so that the A and B curves have their "true" values (and hence will be called the "true" curves of A and B since the death of C reduces its electromotive force approximately to zero). At the start these curves are negative (A more so than B) but on cutting at Z they approach zero. The vertical marks represent 5 second intervals. Selected as typical from 30 experiments.

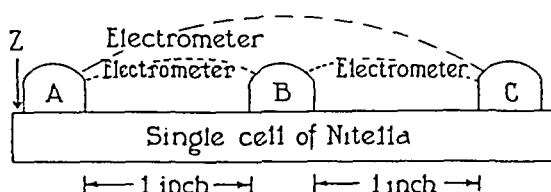


FIG. 6. Like Fig. 4 but with an additional connection between A and B.

form are over: A and B are then negative and have their "true" values (*i.e.* the values observed when the electromotive force of C

⁴ These will not be discussed here since they have been described in a previous paper,¹⁴ where it is shown that the injury at C does not immediately spread to A and B.

is zero). After cutting they approach zero,⁵ the process at *A* being a little in advance⁶ of that at *B* (this is not evident in Fig. 5 but is shown by other experiments).

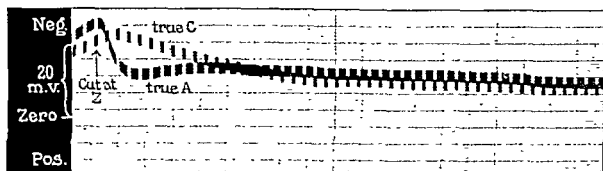


FIG. 7 a.

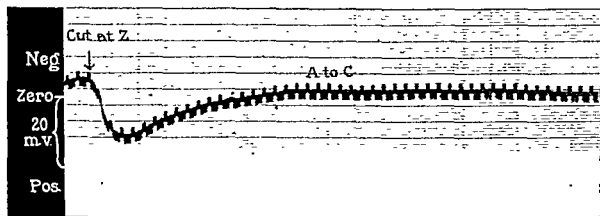


FIG. 7 b.

FIGS. 7 a and b. Photographic records of potential differences, the experiment being arranged as in Fig. 6 with 0.05 M KCl at *A*, *B*, and *C*. The records begin after *B* has been killed (by 0.05 M KCl saturated with chloroform) so that the curves of *A* and *C* show their "true" values (both are negative at the start, *A* being more so than *B*, but on cutting at *Z* they approach zero): these are recorded on one instrument while the curve showing the potential difference between *A* and *C* is simultaneously recorded on another as shown in Fig. 7 b (the slight vertical movements in this curve are due to the alternations of the rotary switch in the other instrument); the value of each point of the curve is equal to the difference between the "true" *A* and "true" *C* values. The vertical marks represent 5 second intervals. Selected as typical from 20 experiments.

⁵ The curves for *A* and *B* are recorded simultaneously by means of the rotating switch previously described (Osterhout, W. J. V., and Harris E. S., *J. Gen. Physiol.*, 1927-28, xi, 417).

⁶ This permits us to study the speed of propagation of the stimulus, which will be discussed in a later paper.

It would seem that leading off from A to B must be similar to leading off from A to C and that the A to C curve must resemble the A to B curve and must be approximately⁷ equal to the difference between the "true" curves of A and C (and hence of A and B in Fig. 5) which would give a curve similar to that of Fig. 2. That this is really so became evident as soon as it was possible, by making simultaneous records on two instruments and using the arrangement shown in Fig. 6, to get the A to C curve together with the "true"

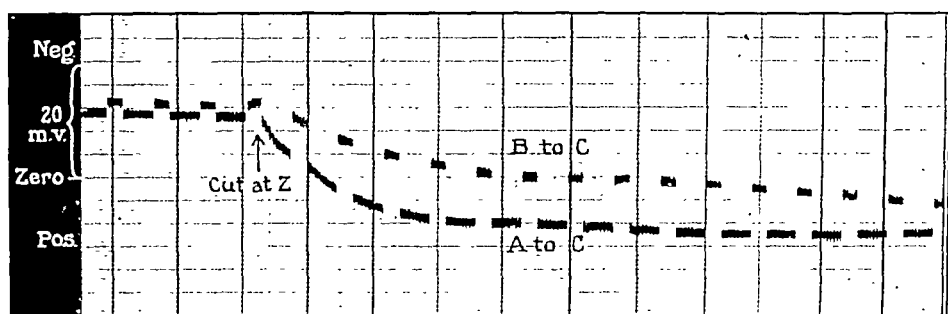


FIG. 8. Like Fig. 5 except that C is not killed. The A and B curves do not have their "true" values (as in Fig. 5) but merely show their potential difference against C . The difference between them, however, gives the same sort of curve as that shown in Fig. 2. The vertical marks represent 5 second intervals. Selected as typical from 30 experiments.

curves of A and C . Inspection of Figs. 7*a* and *b* shows that the difference between the true curves of A and C gives the observed A to C curve, *i.e.*, if we measure the vertical distance between A and C we obtain the vertical distance above or below zero of the A to C curve.

In this case we notice that the death wave which starts at Z must pass over the freshly killed protoplasm at B before it can affect C , but it can evidently do this, as shown by the behavior of C .

To ascertain whether the killing of C modifies the A and B curves we

⁷ We should not expect to get the exact difference since, as shown in a former paper, we do not necessarily measure exactly the same fraction of the true E. M. F. in both cases. But this would not noticeably affect the form of the curves and might make very little quantitative difference between them.

have performed the experiment without killing *C*, the result being shown in Fig. 8. In this case the curves are negative at the start (usually they are near zero under these circumstances) but on cutting

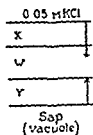


FIG. 9.

FIG. 9. Hypothetical diagram to illustrate the condition of the protoplasm in contact with 0.05 M KCl. The direction in which the positive current tends to flow is shown by the direction of the arrows, the relative magnitude of the electromotive force being indicated by their length. The potential difference across the protoplasm is by convention regarded as negative since the *X* arrow is longer.



FIG. 10 a.

FIG. 10 a. Tracing of the "true" *A* curve in Fig. 5 with certain ordinates drawn for comparison with Fig. 10 b.

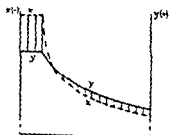


FIG. 10 b.

FIG. 10 b. Theoretical interpretation of Fig. 10 a. Fig. 10 a shows only the observed potential difference, but Fig. 10 b indicates that this is equal to the difference between the (hypothetical) value of *x* (broken line) with negative sign (as shown by the scale of ordinates at the left) and the (hypothetical) value of *y* (unbroken line) with positive sign (as shown by the scale of ordinates on the right): each ordinate is equal to the one directly above it in Fig. 10 a.

Since the potential difference across the protoplasm in contact with 0.05 M KCl is negative the *x* ordinate is made longer at the start (since *x* is negative by convention). When *x* falls and becomes equal to *y* the curve in Fig. 10 a falls to zero: as *x* continues to fall and becomes less than *y* the curve in Fig. 10 a becomes positive; when the two curves approach each other the curve in Fig. 10 a approaches zero.

Since we do not know the absolute value of the ordinates but only the value of the difference between them (*i.e.* the value of the shaded area) the scales of ordinates are interrupted toward the base by a dotted line to signify that below this point the ordinate extends for an indefinite distance. It should be noted that when the *x* and *y* curves come together the observed potential difference (such as is given in Fig. 10 a) is zero but this zero has no relation to the absolute zero of the present figure.

they become positive and then approach zero (in the latter part of the record which is not shown here). The difference between the *A* and

and B curves would evidently give a curve similar to that in Fig. 2. This is to be expected since if we designate the "true" values at A , B , and C by a , b , and c we may write as an approximation

$$\text{Observed } A \text{ to } C \text{ curve} = a - c$$

$$\text{Observed } B \text{ to } C \text{ curve} = b - c$$

Taking the difference between these two curves we have

$$\text{Observed } A \text{ to } B \text{ curve} = (a - c) - (b - c) = a - b$$

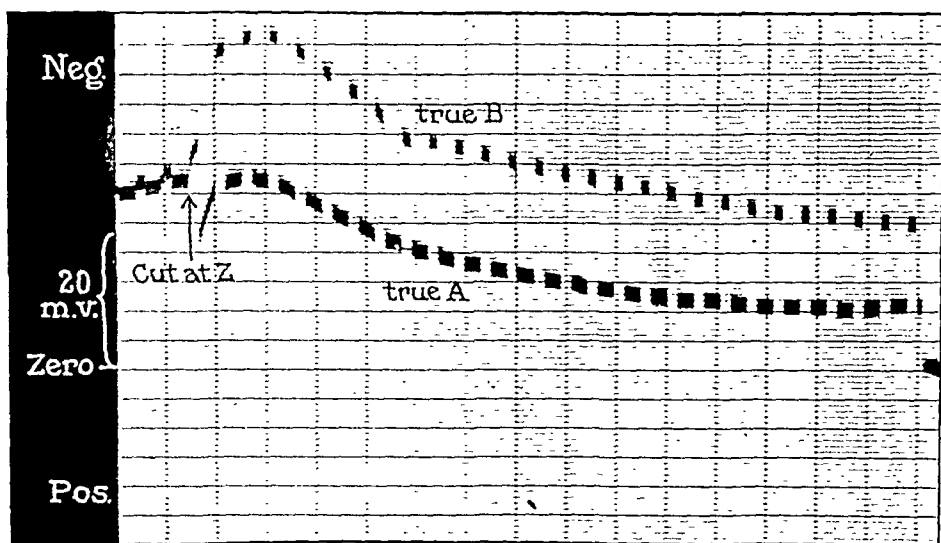


FIG. 11. Photographic record of potential differences (the experiment being arranged as in Fig. 4 with 0.05 M KCl at A , B , and C). The record begins after C has been killed (with 0.05 M KCl saturated with chloroform) so that the curves for A and B show their "true" values and are in consequence negative (their values are so nearly equal that they almost coincide). On cutting at Z , A becomes more positive, then more negative, and then approaches zero (resembling an effect often found with 0.1 M KCl): B becomes more negative and then approaches zero (resembling an effect commonly produced by 0.05 M KCl plus chloroform). The vertical lines represent 5 second intervals. Selected as typical from 20 experiments.

In other words we get the observed A to B curve by taking the difference between the "true" curves of A and B or between the observed curves of A to C and B to C .

The experiments indicate that the killing of *C* does not modify the *A* and *B* curves if the cut is made soon enough after the death of *C*.

Our next step is to try to interpret the "true" curves shown in Fig. 5. These resemble in many cases the "true" curves produced by applying chloroform directly to *A* and *B* (instead of producing injury at *A* and *B* by cutting at *Z*). Such curves have been explained^{1b} on the ground that the protoplasm consists of an outer layer, *X*, and an inner layer, *Y* (both of which are probably non-aqueous), with an aqueous layer, *W*, between them and that the layer in contact with the more

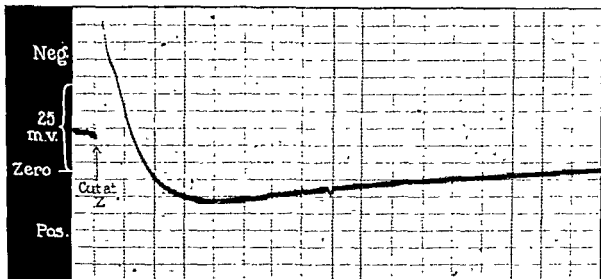


FIG. 12. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.001 M KCl at *A* and *C*. At the start the curve shows a negative value of about 10 millivolts; ordinarily this would be near zero. On cutting at *Z*, *A* becomes more negative and then positive with respect to *C*; the potential difference then approaches zero. The vertical lines represent 5 second intervals. Selected as typical from 20 experiments.

concentrated solution is the first to change. On this basis we may diagram the protoplasm as in Fig. 9, the arrows indicating the direction in which the positive current tends to flow and their length the relative magnitude (the direction of the *X* arrow is called negative by convention and that of the *Y* arrow positive; and since in this case the *X* arrow is longer the potential difference across the protoplasm is here said to be negative). If the value of the *X* arrow is x and that of the *Y* arrow is y we may assume that the observed potential difference is proportional to $y - x$.^{1b} If both these values should fall off simul-

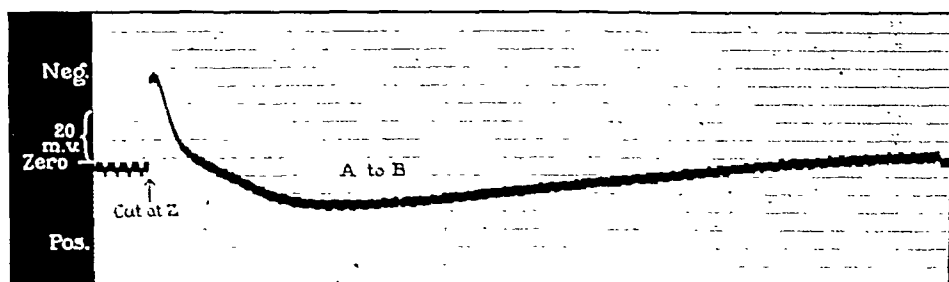


FIG. 13 a.

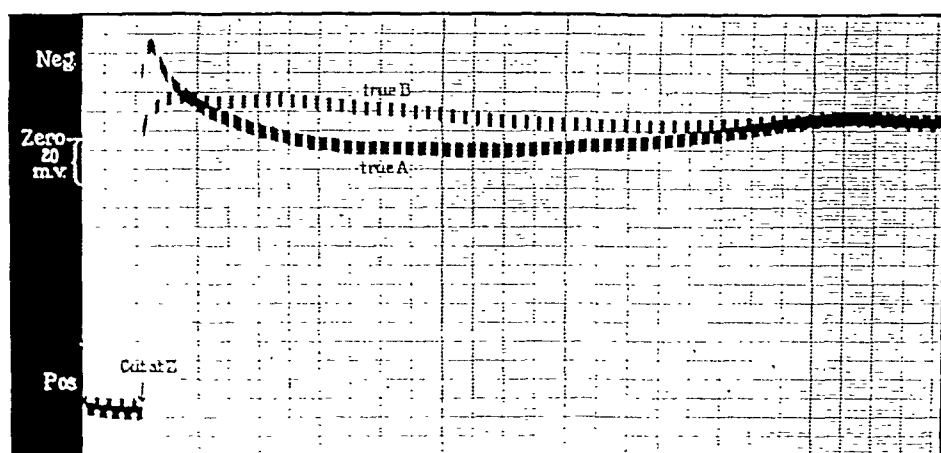


FIG. 13 b.

FIGS. 13 a and b. Photographic record of potential differences, the experiment being arranged as shown in Fig. 6 with 0.001 M KCl at A, B, and C. The record starts after C has been killed (by 0.001 M KCl saturated with chloroform) so that the curves for A and B have their "true" values (both are strongly positive and have about the same value so that they almost coincide; this high value is partly due to the fact that in this material 0.01 M is positive instead of being negative as usual: they become negative on cutting after which they approach zero); these are recorded on one instrument while the curve showing the potential difference between A and B (marked "A to B") is simultaneously recorded on another; the value of the latter is equal to the difference between the "true A" and "true B" (as illustrated in Figs. 14 a and b). The vertical marks represent 5 second intervals. Selected as typical from 25 experiments.

taneously, as shown in Fig. 10*b*, we should obtain the curve shown in Fig. 10*a* which is a tracing of the "true" *A* curve in Fig. 5.

Our hypothesis states that the layer in contact with the more concentrated solution will be the first to change and since 0.05 M KCl acts much like sap we might expect that when it is applied to the outside of the

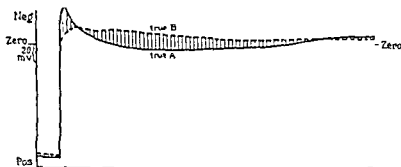
FIG. 14 *a*.FIG. 14 *b*.

FIG. 14 *a*. Tracing of the curve in Fig. 13 *a* with vertical and horizontal scales made identical with those in Fig. 14 *b*. Certain ordinates are drawn for comparison with Fig. 14 *b*.

FIG. 14 *b*. Interpretation of Fig. 14 *a*. Fig. 14 *a* shows only the observed potential difference but Fig. 14 *b* shows that this is equal to the difference between the "true" curves of *A* and *B* (traced from Fig. 13 *b*); each ordinate is equal to the one directly above it in Fig. 14 *a*. At the start *A* is positive to *B* corresponding to the fact that the *A* to *B* curve of Fig. 14 *a* is positive (this curve shows the potential difference of *A* with reference to *B*): when *A* becomes negative to *B* the curve in Fig. 14 *a* becomes negative: *A* then becomes positive to *B* and then as *A* and *B* approach each other the curve in Fig. 14 *a* approaches zero.

cell *X* and *Y* would behave as if in contact with similar solutions and upon cutting would change at about the same rate, or that sometimes one and sometimes the other would go faster. This is actually the case: when there is a difference it is usually *Y* which goes first, just as in the case of chloroform,^{1b} so that the potential difference across the protoplasm first becomes more negative and then approaches zero (curve *B*, Fig. 11). Occasionally it would seem that *X* goes first,

giving curves like the *A* curve shown in Fig. 22*a* (see curve *A*, Fig. 11).⁸

Let us now consider the results obtained with a solution less concentrated than sap, e.g. 0.001 M KCl. We find (Fig. 12) that the cut makes *A* negative, then positive, after which the potential difference approaches zero (Fig. 12). When the experiment is arranged as in Fig. 6 we observe (Figs. 13*a* and *b*) after killing *C* that *A* and *B* are

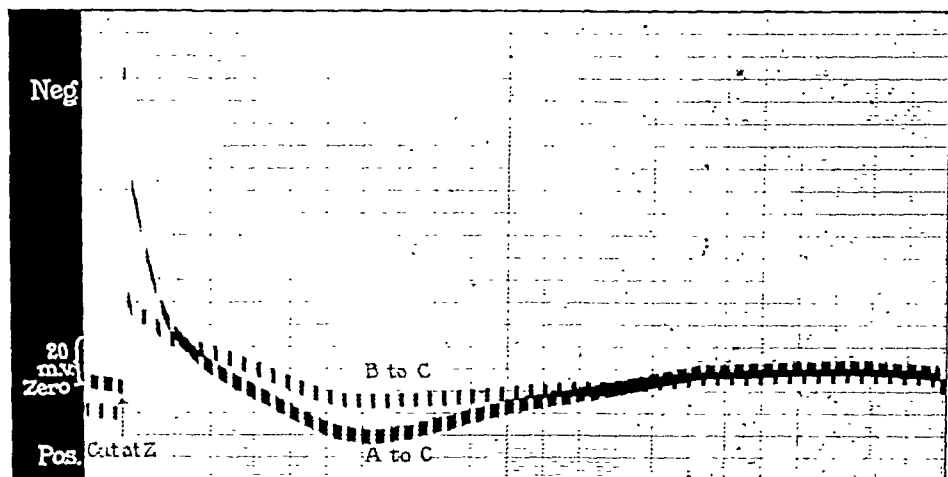


FIG. 15. Photographic record of potential differences, the experiment being arranged as in Fig. 4 with 0.001 M KCl at *A*, *B*, and *C*. On cutting at *Z* the *A* to *C* and *B* to *C* curves become negative, then positive, and the potential difference then approaches zero (the curves do not show the "true" values but only the potential difference with reference to *C*). The vertical lines represent 5 second intervals. Cf. Fig. 5. Selected as typical from 20 experiments.

positive (showing their "true" values).^{1b} On cutting both become negative, after which they approach zero. It is evident that if we should lead off from *A* to *B* we should obtain approximately the difference between the "true" curves, giving a curve of the type seen in Fig. 12. This is the case, as seen in Fig. 13*a* and shown more clearly by Figs. 14*a* and *b*.

⁸ The same cell may show both types of curves (as in Fig. 11) but in many cases but one is observed. The resulting *A* to *B* curves may resemble those shown in Figs. 2, 7 *b* or 22 *b*. The variations may be due to differences in the sap or in the protoplasm.

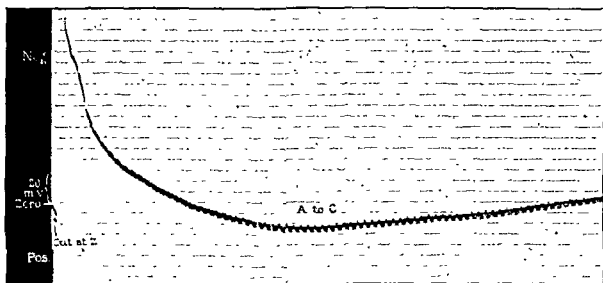


FIG. 16 a.

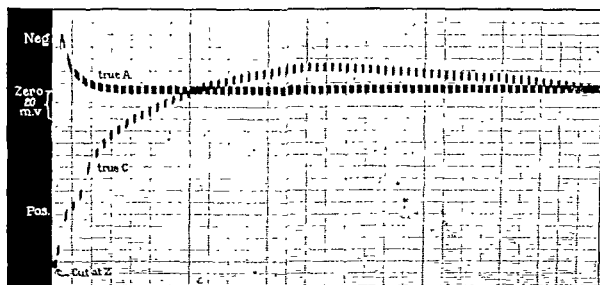


FIG. 16 b.

FIGS. 16 a and b. Photographic record of potential differences, the experiment being arranged as in Fig. 6 with 0.001 M KCl in contact with A, B, and C. The record starts after B has been killed (by 0.001 M KCl saturated with chloroform) so that the curves for A and C have their "true" values: before cutting both have about the same positive value (this is very high corresponding to the fact that with this material 0.01 M KCl is positive instead of being negative as usual) and coincide but after cutting they become negative and then approach zero; these are recorded on one instrument while the curve showing the potential difference between A and C (marked "A to C") is simultaneously recorded on another: the value of the A to C curve is equal to the difference between the "true A" and "true C" (cf. Figs. 14 a and b). The vertical lines represent 5 second intervals. Selected as typical from 20 experiments

If we should perform the experiment without killing *C* it would still be true (as previously explained) that the difference between the curves of *A* and *B* should give the curve observed in leading off from *A* to *B*. The result of such an experiment is shown in Fig. 15 and it is evident

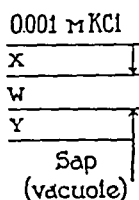


FIG. 17. Hypothetical diagram of the condition of the protoplasm when in contact with 0.001 M KCl. The value of the potential difference across the protoplasm is regarded by convention as positive since the *Y* arrow is longer.

FIG. 17.

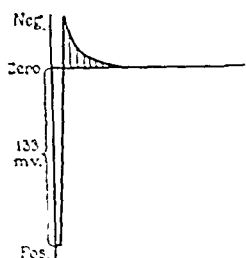


FIG. 18 a.

FIG. 18 a. Tracing of the "true *A*" curve shown in Fig. 16 b. Certain ordinates are drawn for comparison with Fig. 18 b.

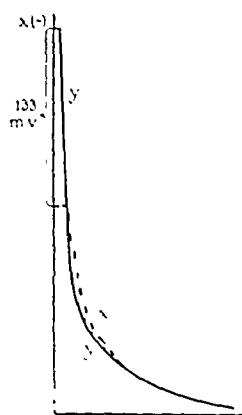


FIG. 18 b.

FIG. 18 b. Interpretation of Fig. 18 a. Fig. 18 a shows only the observed potential difference but Fig. 18 b shows that this is equal to the difference between the (hypothetical) value of *x* (broken line) which has a negative sign and the (hypothetical) value of *y* (unbroken line) with a positive sign: each ordinate is equal to the one directly above it in Fig. 18 a.

At the start the curve in Fig. 18 a is positive because the value of *y* (which is positive by convention) is greater. During the first period *y* falls until equal to *x* and the curve in Fig. 18 a is then at zero: when *y* becomes less than *x* the curve in Fig. 18 a becomes negative; the two curves then come together as the curve in Fig. 18 a approaches zero.

that if we pursue the scheme shown in Figs. 14a and b we shall have a similar result. In order to get the "true" *C* curve we employ the scheme shown in Fig. 6 and obtain the result in Figs. 16a and b (the *C* curves show considerable variation which will be discussed in a subsequent paper).

The next step is to interpret the "true" curves in 0.001 M KCl, such as the A curve in Fig. 13*b*. For this purpose we may diagram the protoplasm as in Fig. 17 making the I' arrow longer in order to show that the potential difference across the protoplasm is positive. If cutting

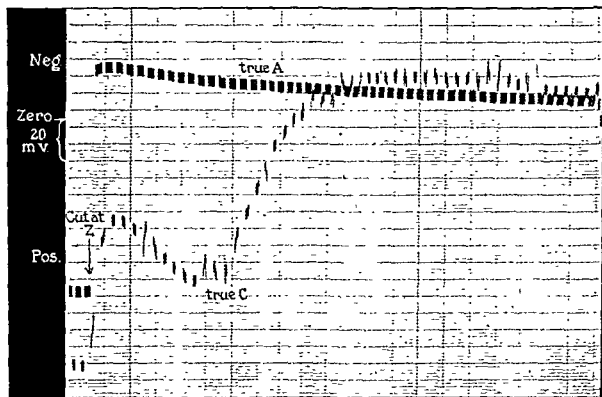


FIG. 19. Photographic record of potential differences, the experiment being arranged as shown in Fig. 6 with 0.001 M KCl at A , B , and C . When the record starts B has been killed (with 0.001 M KCl saturated with chloroform) so that the curves for A and C show their true values, C being more positive than A (the high positive values correspond to the fact that with this material 0.01 M KCl is positive instead of being negative as usual). On cutting at Z the curve of A becomes negative and then approaches zero: that of C becomes more negative, then more positive, then more negative, and then approaches zero (resembling curves obtained by killing with chloroform). The vertical lines represent 5 second intervals. Selected as typical from 20 experiments.

caused the value of y to fall off more rapidly than that of x we might get the curve shown in Fig. 18*a* (which is a tracing of the "true" A curve of Fig. 16*b*). This would be expected on the basis of our hypothesis which states that in general the changes produced (by chloroform or by cutting) in any protoplasmic layer are more rapid the

higher the concentration of the salt solution in contact with it. As sap is approximately equivalent in these experiments to 0.05 M KCl it is

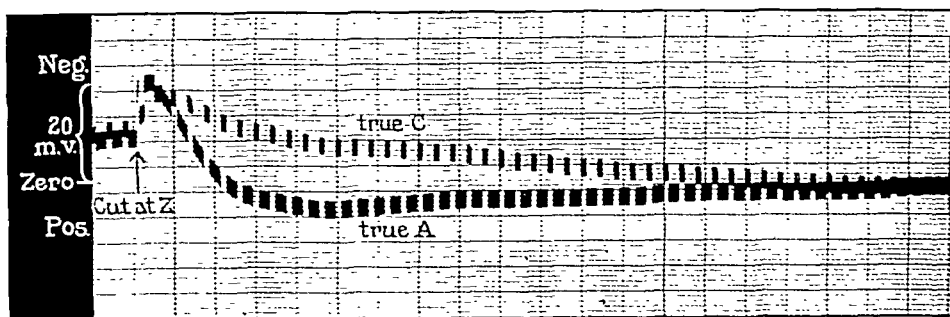


FIG. 20 a.

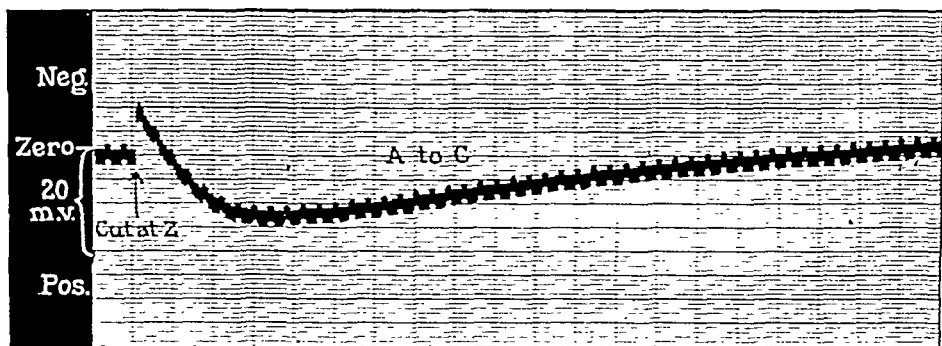


FIG. 20 b.

FIGS. 20 a and b. Photographic record of potential differences, the experiment being arranged as in Fig. 6 with 0.025 M KCl in contact with A, B, and C. The record starts after B has been killed (with 0.025 M KCl saturated with chloroform) so that the curves for A and C have their "true" values (both are negative at about the same value so that they almost coincide). On cutting at Z they become more negative and then approach zero (the process being more rapid at A than at C); these are recorded on one instrument while the curve showing the potential difference between A and C (marked "A to C") is simultaneously recorded on another, the value of the A to C curve is equal to the difference between the "true A" and "true C" (cf. Figs. 14 a and b). The vertical lines represent 5 second intervals. Selected as typical from 20 experiments.

evident that in this case we should expect I^{1b} which is in contact with sap to go before A which is in contact with 0.001 M KCl.

When we compare the "true" curves in Fig. 13*b* with those obtained (as described in a previous paper¹⁶) by applying chloroform directly to *A* and *B* (instead of injuring them indirectly by cutting at *Z*) we see a general resemblance but they differ in details.⁹ All that we can say at present is that the forms of these curves seem to depend on the relative rates of change of *x* and *y* without attempting to explain why cutting in many cases produces effects which are somewhat different from those observed with chloroform. (An example of another type

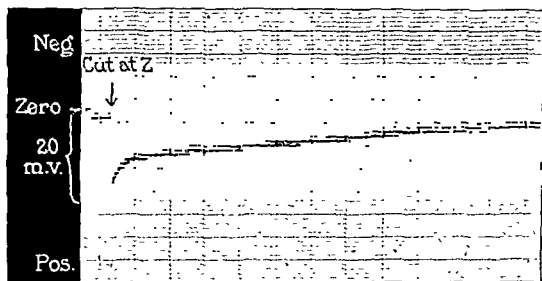


FIG. 21. Photographic record showing potential differences, the experiment being arranged as in Fig. 1 with 0.1 M KCl in contact with *A* and *C*. On cutting at *Z* the curve showing the potential difference of *A* with respect to *C* becomes more positive and the potential difference then approaches zero. The vertical lines represent 5 second intervals. Selected as typical from 30 experiments.

The first mark is a zero mark.

of curve commonly found with chloroform is shown in the *C* curve in Fig. 19.¹⁰)

Let us now consider concentrations (0.01 and 0.025 M KCl) which have a special interest because in some cases they produce a negative potential difference across the protoplasm. Our hypothesis predicts that in spite of this they will act on cutting like 0.001 M KCl which (prior to making the cut) shows a positive potential difference across the protoplasm. The following considerations show why this is so.

⁹ Foot-note 1*b*, Fig. 12 *a*.

¹⁰ Foot-note 1*b*, Fig. 9.

Since sap acts like 0.05 M KCl, 0.01 and 0.025 M KCl are less effective¹¹ solutions and we therefore expect the layer *Y* (which is in contact with sap) to go first; this would at first make the protoplasm more nega-

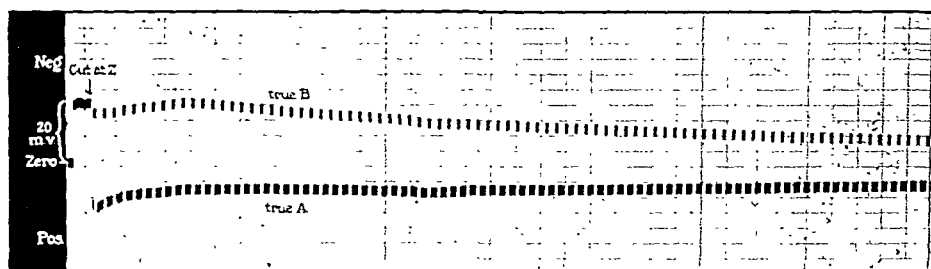


FIG. 22 a.

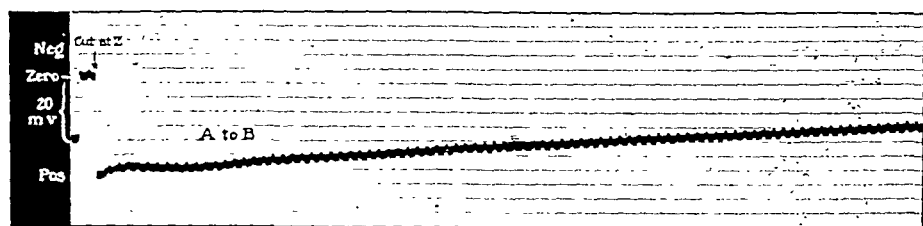


FIG. 22 b.

FIGS. 22 a and b. Photographic record showing potential differences, the experiment being arranged as in Fig. 6 with 0.1 M KCl at *A*, *B*, and *C*. The record starts (the first mark in Fig. 22 a is a zero mark and the first mark in Fig. 22 b is a calibration mark denoting 20 millivolts) after *C* has been killed (by 0.1 M KCl saturated with chloroform) so that the curves for *A* and *B* have their "true" values (both have the same negative value so that they coincide: on cutting they approach zero, *A* first becoming positive); these are recorded on one instrument while the curve showing the potential difference between *A* and *B* is simultaneously recorded on another (marked "*A* to *B*"); the value of the latter is equal to the difference between the "true *A*" and "true *B*." The vertical marks represent 5 second intervals. Selected as typical from 30 experiments.

tive (just as in the case of 0.001 M KCl). Fig. 20a shows that this is the case.

The interpretation of the "true" curves in Fig. 20a is like that of the curves obtained with 0.001 M KCl (*cf.* Figs. 18a and b).

¹¹ The word "effective" is used here in a technical sense. A solution is less effective than sap if it acts like dilute sap as tested by electrical criteria.

There is another case which should be considered, that of a solution more effective than sap (Fig. 21). Since in this case X is in contact with the more effective solution we should expect it to go first. This would make the protoplasm more positive after which the potential



FIG. 23 a.

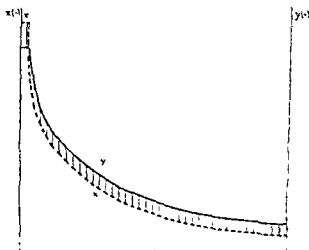


FIG. 23 b.

FIG. 23 a. Tracing of the "true" A curve shown in Fig. 22 a with certain ordinates drawn for comparison with Fig. 23 b.

FIG. 23 b. Interpretation of Fig. 23 a. Fig. 23 a shows only the observed potential difference but Fig. 23 b shows that this is equal to the difference between the (hypothetical) value of x (broken line) which has a negative sign and the (hypothetical) value of y (unbroken line) with a positive sign: each ordinate is equal to the one directly above it in Fig. 23 a.

At the start x is greater than y because the curve in Fig. 23 a is negative (and x is negative by convention); when v falls and becomes equal to y the curve in Fig. 23 a reaches zero; when x becomes less than y the curve in Fig. 23 a becomes positive.

difference would approach zero. That this is the case is shown in Fig. 22a. The behavior of the "true" B curve resembles that found with chloroform but in the case of the "true" A curve the first positive movement of the curve goes further and carries it past zero. The "true" curves show a positive drop at the start after which the potential difference approaches zero (in this case so slowly that only a part of

the curve is given): the process is more rapid and more pronounced at *A* than at *B*.

In this case we diagram the protoplasm as in Fig. 9 (but making the α arrow longer than in the figure) and interpret the "true" *A* curve in Fig. 22*a* in the manner shown in Figs. 23*a* and *b*.

These experiments confirm in a remarkable way the predictions of the hypothesis set forth in a previous paper^{1b} and indicate that it may prove a useful guide.

We see that the injured protoplasm is sometimes positive and sometimes negative to uninjured protoplasm because the electromotive force of the dead protoplasm is nearly zero and its relation to normal protoplasm will depend on what solutions are employed. By making the concentration of KCl 0.01 or thereabouts we can always bring the living protoplasm of *Nitella* to the same potential difference as the dead protoplasm. If we use more concentrated KCl the injured spot will be positive to the uninjured one and with more dilute solutions it will be negative.^{1c}

Certain interesting phenomena connected with the death wave, *e.g.* the fact that it traverses the cell so rapidly and can pass over a killed spot (though unable to affect adjoining cells) as well as the fact that the death process is more rapid and more pronounced the greater its nearness to the cut, will be discussed in forthcoming papers.

SUMMARY.

Experiments on cutting confirm the prediction that the current of injury will be positive when the cell is in contact with concentrated solutions and negative with dilute solutions. They support the idea that the protoplasm is made up of layers differing considerably in their properties, each having a death curve of simple and regular form, the more rapid alteration of the outer layer making the protoplasm more positive and the more rapid alteration of the inner making it more negative.

From the point where the cell is cut a wave of some sort, which we may for convenience call a death wave, passes along the cell, setting up at each point it touches a death process which has the greater speed and intensity the nearer it is to the cut.

^{1c} *i.e.* always using identical solutions at both the injured and uninjured spots: otherwise there may be an effect due to the cell wall (concentration effect).

ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life, and appeared simultaneously with Volumes IX, X, and XI.

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MEMBRANES FOR ULTRAFILTRATION, OF GRADUATED FINENESS DOWN TO MOLECULAR SIEVES.

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It is becoming increasingly evident that ultrafiltration is amongst the most important methods of colloidal and biological investigation. In many instances ultrafiltration is the chief test for the presence or existence of substances in the colloidal state. In medicine it is becoming an indispensable tool in the study and control of filter-passing viruses and in physical chemistry new fields of investigation of the constitution of aqueous and non-aqueous solutions open out with the advent of graded molecular sieves. For all these reasons it is desirable that trustworthy membranes should be universally familiar.¹

For many years one of us has advocated the use of sheets of cellophane for use in ultrafiltration. They possess the advantage of being obtainable commercially in large sheets or batches which are approximately uniform in their properties.² The material of which they are composed is as non-reactive as filter paper consisting of cellulose with a trace of glycerol.³ Cellophane may be obtained in various forms but

* With addenda by M. C. Field.

¹ An example of the many almost unexplored possibilities of the quantitative use of ultrafiltration may be seen in a previous paper by McBain and Jenkins (McBain, J. W., and Jenkins, W. J., *J. Chem. Soc.*, 1922, cxxi, 2325) where it is applied to the study of soap solutions measuring not only amounts of colloid and crystalloid present but also hydrolysis, osmotic pressure, hydration or solvation of the material held back by the ultrafilter and even relative proportions of the different kinds of colloidal particles.

² For example, they are now manufactured by the E. I. du Pont de Nemours and Company.

³ In filtering soap solutions through collodion membranes obtained from collodion dissolved in glacial acetic acid we found that there was an appreciable proportion of cellulose acetate in the membrane which entered into double decomposition with the soap and distorted certain of the quantitative measurements.

the flat sheets are especially convenient for clamping in ultrafilters which may be operated under high pressures⁴ up to 100 atmospheres. Cellophane is much stronger than collodion whilst the latter, under high pressures, tends to become embedded in the very fine bronze gauze which forms the most suitable support and therefore becomes difficult to replace by a fresh membrane. Cellophane which has been swollen

TABLE I.

Rates of Filtration of Various Liquids Through "600" Cellophane.

Solution	Pressure used kg./sq. cm.	Filtrate per sq. cm. per hr. per kg. per sq. cm.
Water.....	47	0.02
AgNO ₃ in piperidine.....	17	0.0009
NH ₄ I in aniline.....	15	0.00004
NaBr in C ₂ H ₅ OH.....	75	0.0001
H ₂ O in paraldehyde.....	50	None
H ₂ O in amyl alcohol (iso).....	50	None
AgNO ₃ in (C ₂ H ₅) ₂ NH.....	14	None

in water possesses pores so large that no molecules, but only colloidal particles are held back.

Cellophane is obtainable in several different degrees of porosity as measured for example by the bubble test⁵ and it will be shown that there are several methods of progressively enhancing the fineness of

⁴ The most convenient form of ultrafilter which we have used is that supplied by Vereinigung Göttinger Werke, Göttingen (with or without electrical stirrer) which is attached directly to a cylinder of nitrogen and will stand pressures up to 150 kilos per sq. cm.

⁵ See Bechhold, H., *Z. phys. Chem.*, 1907, lx, 257; also 1908, lxiv, 330, in both of which papers he made a mistake of a decimal point expressing his pore diameters as was pointed out by Bigelow, S. L., and Bartell, F. E., *J. Am. Chem. Soc.*, 1909, xxxi, 1197, and implicitly accepted by Bechhold in his later papers (see Bechhold, H., and Szidon, V., *Kolloid-Z.*, 1925, xxxvi (Ergänzungsband Zsigmondy) 264). The results of the method are not entirely free from contradictions although it is the best available and certainly yields comparative results. It measures only the few largest pores, and much of the filtration must occur through smaller ones. It seems as if a Bechhold ultrafilter holds back particles whose diameter is only about one-fifth of the size of the largest pores.

these membranes until they hold back first the larger crystalloidal molecules and then smaller ones, until the smallest molecules and ions are retained. For example, it is easy to separate by ultrafiltration sucrose from methyl alcohol in aqueous solution. Lastly, it will be shown that these membranes can be used in the study of non-aqueous solutions with such solvents as benzene, amyl alcohol or paraldehyde.

The Permeability and Swelling of Cellophane Membranes in Various Solvents.

Water passes through ordinary cellophane very readily. Piperidine passes through slowly but at a satisfactory rate for ultrafiltration,

TABLE II.
Swelling Produced by Soaking Cellophane "600" in Various Solvents.

Solution	Initial thickness	Thickness in solution after lapse of stated time			
	mm \approx 0.002	3 hrs.	15 hrs.	24 hrs.	150 hrs.
	mm.	mm.	mm.	mm.	mm.
Water.....	0.047	0.098	0.098		
95 per cent C_2H_5OH	0.045	0.047	0.047	0.048	0.048
Amyl alcohol.....	0.045	0.044	0.043	0.044	0.044
Paraldehyde in water (saturated solution).....	0.047	0.090	0.093	0.093	0.093
Aniline.....	0.040		0.042	0.041	0.042

while ethyl alcohol and aniline filter only extremely slowly. Amyl alcohol, paraldehyde and diethylamine pass too slowly to be measured in a reasonable length of time.

Table I gives the rates of filtration for solutions using these solvents. The cellophane used was "600," one of the densest obtainable commercially.

Since Brown (1) has found that the rate of diffusion of substances through a collodion membrane, and also the apparent reticular size, or the porosity, depends directly upon the previous swelling of the membrane it seemed possible that different degrees of swelling of the cellophane produced by contact with the solvent could explain the wide differences in permeability for the different solvents in Table I.

Accordingly swelling experiments were undertaken, the results of which are shown in Table II. It was found that the sheets of cellophane expand to different extents in different directions. Thus whereas it is shown in Table II that the thickness is increased by over 100 per cent by soaking in water the sheet does not expand at all in length, and expands only 9 per cent in width. It is a great advantage that the area of the cellophane is so little affected. The difference in expansion in the two directions is presumably brought about by the squirting through the slit in the factory and might be interpreted as evidence of an incipient fibrous structure. It is seen from Table II that there is a rough correlation between the amount of swelling induced by the solvent and the permeability of the membrane for that solvent.

It was attempted to increase the permeability of the cellophane to non-swelling liquids by previously swelling the membranes in water and then attempting to force the particular solution through. To our surprise, this was of no avail except in the cases of ethyl alcohol, diethylamine and ethylamine. The membranes remained as impermeable to aniline, amyl alcohol and paraldehyde as ever.

In order to keep the membrane in as nearly the same thermodynamic environment as possible throughout the experiment, new trials were made, this time filtering two phase solutions instead of one. For example, water and amyl alcohol were shaken together till both the aqueous and alcoholic layers were thoroughly in equilibrium with each other. In each of the three cases tried the aqueous layer was the denser so that it came into contact with the membrane first. The membrane was swollen in water first before being placed in the ultrafilter. The aqueous layer passed through the membrane rapidly with apparently no change in concentration, with the possible exception of the aniline solution in which it seemed as though some aniline were being retained (analysis unsatisfactory).

As soon as the upper (non-aqueous) phase reached the membrane, filtration ceased. This was to be expected, for it is exactly like the bubble test of Bechhold.⁵ Frequently when a liquid has all gone through a dense ultrafilter the wet membrane will completely hold back the compressed nitrogen. It requires a pressure of 100 atmospheres to blow a bubble through a hole 30\AA . u. ($30 \times 10^{-8}\text{ cm.}$) in diameter if the hole is covered with water. The pressure is inversely

proportional to the diameter of the opening and directly proportional to the surface tension of the liquid. If there are two liquids as in the present case the interfacial tension plays the same rôle as the surface tension of one liquid against nitrogen or air, and if all the holes are smaller than the calculated value the meniscus cannot pass through.

According to this explanation, a membrane saturated with one liquid should be permeable to another liquid that is completely misci-

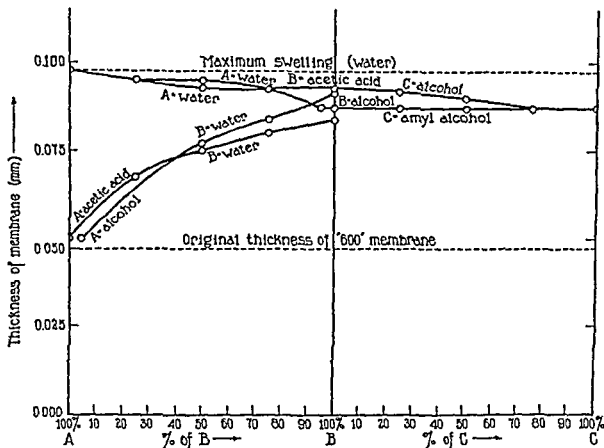


FIG. 1. Thickness of "600" cellophane when placed successively in a solvent A, then in mixtures in which A is replaced by a second solvent B, then in some cases by a third solvent C.

ble with the first. Our observations on the permeability of cellophane, saturated with water, to alcohol and the amines support this view. Since the cellophane becomes permeable to alcohol and the amines by swelling in water; that is, a liquid with which they are miscible, it is possible to make the membrane permeable to the other liquids by using an intermediary liquid such as alcohol with which they in their turn are miscible. Thus a membrane, swollen in water and soaked

successively in 25, 50, 75 and 95 per cent ethyl alcohol and then in several mixtures up to amyl alcohol, proved readily permeable to the latter as well as to benzene, aniline and paraldehyde. It is just as effective to transfer the cellophane directly from water to ethyl alcohol and then to the other liquid except in the case of paraldehyde. Here the final permeability was produced much more quickly by proceeding in stages. Thick membranes always require longer soaking in the ethyl alcohol than do thin ones. Bechhold, in his original paper,⁵ replaced water by alcohol and acetone and used the collodion filters

TABLE III.

Rates of Filtration of Alcohol-Water and Acetic Acid-Water Solutions through Membrane "600" Previously Swollen in Water.

Membrane	Solution	Viscosity at 25°	Pressure	Permeability*
	<i>per cent</i>		<i>kg./sq. cm.</i>	
1	Acetic acid 25	0.024	50	0.000206
	" " 50	0.020	50	0.000103
	" " 75	0.016	75	0.000079
2	Water	0.009	47	0.000097
	Alcohol 25	0.019	53	0.000115
	" 47.5	0.024	50	0.000120
	" 75	0.019	50	0.000100
	" 95	0.014	51	0.000105
	" 75	0.019	50	0.000103
	Water	0.009	53	0.000091

$$* \frac{\text{Filtrate} \times \text{viscosity}}{\text{Time} \times \text{pressure} \times \text{area}}$$

for ultrafiltration of alcoholic chlorophyl. In his paper in 1925⁵ he obtained membranes for use with non-aqueous solvents by coagulating collodion with such solvents, the best instance being collodion dissolved in ether-alcohol for impregnation of filter paper, coagulated by toluene.

Fig. 1 shows the changes in thickness of the membrane which accompany such treatment with various solvents. Clearly, the solvent causing the swelling is water. Neither alcohol nor glacial acetic acid produces appreciable swelling. Once swollen, the cellophane

retains most of its thickness when the water is replaced by another solvent. The slight but distinct shrinkage on replacing water by ethyl alcohol is the same whether the transition is made in stages or directly. The membrane did not reach its full swelling when it was placed first in acetic acid or alcohol and then in water.

In the case of isoamyl alcohol, there seems to be a more pronounced inhibiting effect on the swelling. A membrane that had been soaked in the amyl alcohol and then placed in water did not attain more than 80 per cent of its normal swelling even in several hundred times the time ordinarily required. Bearing on the same point is the observation that the swelling of a membrane soaked in water is reduced

TABLE IV.

Rates of Filtration of 95 Per Cent Alcohol and of Benzene through Specimens of Cellophane "600" (after Swelling in Different Concentrations of Aqueous Alcohol).

Swelling solution	Initial thickness	Thickness in 95 per cent alcohol	Alcohol		Benzene	
			Pressure	Rate*	Pressure	Rate
<i>per cent</i>	<i>mm.</i>	<i>mm.</i>	<i>kg.</i>		<i>kg.</i>	
Alcohol 75	0.060	0.058	38	0.00088	36	0.00018
" 50	0.075	0.070	37	0.0026	31	0.00034
" 25	0.082	0.075	31	0.0039	35	0.00039
Water	0.095	0.082	37	0.0037	38	0.0013

* Cc. divided by pressure (kg./sq. cm.), time (hours) and area (sq. cm.).

approximately 35 per cent by transferring it to a saturated solution of amyl alcohol in water, whereas if the membrane is first transferred to 95 per cent ethyl alcohol from the water and then placed in the amyl alcohol, the swelling is reduced only about 20 per cent.

The results in Table III show that swelling does not alone determine the permeability of the membrane. The last column is the value for the amount of filtrate multiplied by viscosity and divided by time, pressure and area.

On the other hand Table IV shows that swelling has some association with permeability. Different pieces of cellophane showed a permeability to alcohol and thereafter to benzene that increased rapidly with amount of the initial swelling.

Cellophane that is thoroughly washed and afterwards dried at 100° is hard and brittle, the same is true if the drying is conducted over sulfuric acid at room temperature. A membrane that is washed thoroughly with water and then placed in 95 per cent alcohol and then in amyl alcohol and allowed to dry at room temperature was not brittle but was completely impermeable to amyl alcohol. Another similar membrane transferred from the amyl alcohol to benzene before it was dried was impermeable to benzene. This is a great contrast to the experiments described in connection with Fig. 1 where the membrane is not dried.

Preparation of Dense Ultrafilters.

Cellophane swollen in water allows all such undoubted simple molecules as sucrose to pass through freely so that the filtrate has the same composition as the original liquid. However it does hold back the larger colloidal particles. Cadmium iodide dissolved in amyl alcohol is probably an example of a non-aqueous colloidal electrolyte, judging by its migration number. Upon filtering it through swollen cellophane the solution above the membrane became distinctly more concentrated to the extent of 4 per cent. A membrane originally swollen in 50 per cent ethyl alcohol was evidently somewhat denser, for the solution of cadmium iodide above the membrane was increased in concentration to 144 per cent of its original value. For a systematic investigation of this field of physical chemistry in aqueous and non-aqueous solutions denser membranes had to be developed. Bechhold was unsuccessful in his attempts to make such ultrafilters but several authors have described very dense membranes which however they have not used for actual ultrafiltration (see for example Northrop, J. H., *J. Gen. Physiol.*, 1928, xi, 233; and Michaelis, L., *Colloid Symposium Monograph*, 1928, v, 135). It must be strongly emphasized that sieve action or ultrafiltration bears no relation to thermodynamic calculations based upon equilibrium or to osmosis anomalous or otherwise, or to diffusion or to electroosmosis. This distinction is almost invariably lost sight of.

Clogging of the membrane may take place when filtering a solution, and it occurred to us that it might be possible to control the permeability of a membrane by purposely clogging it with some insoluble

substance.⁶ A highly dispersed colloid would seem to be the best suited for the purpose. Accordingly solutions of cellulose in Schweitzer's reagent were filtered through the cellophane, cellulose being preferred because of its insolubility in the liquids under investigation. A small quantity of the cellulose solution diluted with water and filtered through the membranes greatly reduced their permeability. The reduction in permeability can be fairly well controlled reasoning from our few experiments. Moreover the rate of filtration remains satisfactory even when molecules such as sucrose are being held back.

A troublesome difficulty met with was the tendency of the bronze gauze backing for the membrane in the ultrafilter to dissolve in the ammoniacal solution. Gold plating the gauze proved to be a protection for several filtrations, but eventually failed. As a consequence, the stock solution of cellulose had to be diluted by 5 or 10 times its original volume with water before being used. The stock solution was made by dissolving 5 gm. of c.p. copper carbonate in 100 ml. of concentrated ammonium hydroxide and saturating with cellulose.

The permeability of the membrane decreased very rapidly with the first small quantity of cellulose filtered and continued to decrease with more solution but at a slower and slower rate apparently approaching a minimum when relatively small amounts of cellulose had been deposited. Probably the rapid decrease at first can be accounted for by the clogging of the larger openings in the reticular structure. The probabilities are that the imbedded membrane produced by filtering a colloid out of solution is much more uniform than the untreated cellophane. When the larger openings have been closed, the additional decrease in permeability effected by further depositing the cellulose would come mainly from the added friction to the passage of the solution and from the reduction of all of the openings to the average size of the superimposed layer.

Observations on films of collodion have led us to believe that more

⁶ McBain and Jenkins¹ used such clogging of the pores as evidence that the so called emulsoids are not emulsions because true emulsions do not clog the pores. Bigelow and Bartell⁵ have clogged pores of porcelain with barium sulfate and sulfur but that was on a much coarser scale. Much earlier Martin (Martin, C. J., *J. Physiol.*, 1896, xx, 364) prepared ultrafilters by impregnating the wall of a Pasteur-Chamberlain filter with gelatin or silicic acid.

concentrated solutions of cellulose produce much smaller limiting permeability. Another method of decreasing the permeability is by filtering solutions of collodion in alcohol-ether through a membrane that has been previously swollen in water and then transferred to alcohol. The result is to deposit and embed upon the upper surface of the cellophane an exceedingly thin film of collodion. The range of permeabilities extends down to the completely impermeable condition.

The larger the percentage of alcohol in the collodion, the greater the permeability of the treated cellophane as measured by the rate of filtration in pure water. There seems to be a compacting of the membrane after the solvent has filtered through if the pressure is left on for several hours. The densest membranes that we have made have been made with collodion in 50 per cent ether in alcohol solvent and allowed to compact after the solvent has passed through. Such a membrane even when the collodion is not over 0.007 mm. thick is practically completely impermeable to water. A membrane of this kind will pass a strong alcohol solution, and thereafter it proves to be more permeable to water, the permeability depending upon the percentage of alcohol in the solution that was filtered. If an alcohol-water solution is allowed to stand over a collodion membrane, made as above, without pressure for an hour or two and then replaced with water it is found to have had a much greater effect in increasing the permeability. It seems quite evident from this fact that the collodion in the alcoholic solution is soft enough to be compacted by the pressure. Replacing alcohol solution with water probably hardens the collodion enough to make it resist deformation.

The remaining observations in this paper were made by M. C. Field at Bristol University, Mr. W. F. K. Wynne-Jones having previously shown that sucrose solutions passed unchanged through the densest membranes formed by the deposition of nickel on phosphor bronze as described by Manning, *J. Chem. Soc.*, 1926, 1327. Mr. Field found that the densest membranes of cellophane and membranes of cellulose acetate likewise had no effect, behaving in this respect like filter paper or bacteriological filters.

Collodion Membranes.

The general method used in their preparation was as follows: 9 cc. of 14 per cent collodion in 50/50 ether-alcohol as specially sup-

plied through the kindness of the British Xylonite Company were diluted by the addition of a further 27 cc. of 50/50 ether-alcohol, making a total of 36 cc. of 3.5 per cent collodion in ether-alcohol. This is then poured into the lower half of a desiccator (diameter $4\frac{1}{2}$ to 5 inches) containing clean mercury, and allowed to stand for several hours until set. When sufficient solvent has evaporated, the membrane is removed by cutting round the edges with a penknife and lifting from the mercury surface. It is then placed, between filter papers to shrink for a definite time, the final permeability depending to a large extent on the time which elapses during this period of shrinking.

The membrane is finally placed under water for 24 hours and is then ready for use. The method used to change the permeability, which had been employed up to the present, depends on the fact that the permeability increases when the membrane is soaked in alcohol-water mixtures. Therefore, by allowing the membrane to shrink until it is quite impermeable to even water molecules and then soaking it in a definite alcohol-water mixture, a standard type of membrane can be obtained (see (1) pages 40, 591). This method has been used by Mr. Field. The higher the percentage of alcohol in the alcohol-water mixtures the more permeable is the resulting membrane. Also the variation in permeability is very much greater for high percentage alcohol mixtures than for low ones, *i.e.* alcohols of say 10 to 60 per cent do not differ in their action on the membrane so greatly as alcohols of from 90 to 95 per cent.

Thus the apparent pore size of any undried collodion membrane depends only on the per cent of alcohol in the solution in which it is soaked. That is to say, variation in thickness of the membrane in the time taken to make and in the time taken to dry, or in the per cent collodion in the original solution, makes no difference to the proportions of the constituents in a solution held back by the membrane, so long as it is soaked for a sufficient length of time in the alcohol solution. Two such similar membranes will let through a filtrate of the same composition provided that both are allowed enough time, even where the time required for the actual filtration differs many fold.

For all practical purposes, the effect of soaking in alcohol is completed in 12 hours. After that length of time only inappreciable alterations in the permeability take place.

In the later work, using these collodion membranes a somewhat different method of preparation was used. 60 cc. of 3.5 per cent collodion in 50/50 ether-alcohol were poured into a fairly shallow dish on to a surface of mercury ($7\frac{1}{2}$ inches in diameter) and allowed to set, when it shrinks from the sides of the vessel, to which it adheres for some time after it has set, it is removed from the surface and placed between blotting paper until completely dried out. The permeability is then adjusted by the per cent alcohol in an alcohol-water mixture in which it is soaked preparatory to placing in the ultrafilter.

Brown's method (1) for preparing a 10 per cent shrinking membrane by pouring 8 per cent collodion in 50/50 ether-alcohol over a clean glass plate and setting by immersion in water, was repeatedly tried with little success. The membranes invariably contained bubbles which made them quite unsuitable for high pressure ultrafiltration work.

One thick collodion membrane was obtained after soaking in 60 per cent alcohol which held back sucrose in aqueous solution giving almost pure water as filtrate and showing no clogging of the pores with time.

Attempts were next made to repeat this result with a second membrane prepared in exactly the same manner. This, however, could not be done; a large number of membranes were prepared but their chief feature was one of variability. Thus, sometimes the above semipermeability was obtained and in other cases, the membrane was either completely impermeable or completely permeable to both sugar and water. All states between these limits were obtained.

A membrane which under 100 atmospheres pressure allowed 2 cc. of water to come through from $N/2$ sucrose for 24 hours allowed only 3 drops of 25 per cent aqueous alcohol to pass through in a week, although the aqueous alcohol was unchanged. Such membranes, holding back large proportions of sucrose, likewise hold back large proportions of potassium chloride, which may even crystallize out in solid form as the residue becomes concentrated. However, from a quantitative standpoint no two results are obtained alike and the collodion membranes appear to alter whether they are allowed to dry and then res soaked in alcohol, or whether they are kept beneath water, or in their standard alcohol-water mixture.

Animal Membranes—Pig's Bladder.

Gold beater's skin (see Bigelow and Gemberling (3)), Bedicher (a membrane obtained from the cow's intestine) and pig's bladder membranes were each experimented with. The latter are the only ones to give any satisfaction as molecular sieves although Bedicher holds back a slight amount of sucrose. The Bedicher is very fine, like tissue paper, and filters very rapidly and should be very useful with fine colloids. The membranes were prepared by inflating the pig's bladder in the usual manner and allowing it to dry until brittle. Different types of membranes were obtained according to the extent of inflation. The greater the tension the greater the porosity. Once obtained their properties remain practically constant. These membranes filter very much more rapidly than collodion.

Pig's bladder holds back only $\frac{1}{2}$ to $\frac{3}{4}$ of the sugar in aqueous sucrose. It also holds back a portion of potassium chloride, and to a less extent glycerol and urethane from their aqueous solutions. In each case of course the residue becomes more concentrated. The filtrate from aqueous ethyl and methyl alcohol is likewise somewhat diluted.

Bechhold (Bechhold, H., *Z. phys. Chem.*, 1907, 1x, 262) tested parchment paper, fish bladder and amnion membrane and found the fish bladder most uniform though not as uniform as his artificial membrane.

SUMMARY.

The use of cellophane in ultrafiltration is recommended. It is shown that after it has been swollen in water it does not hold back molecules such as sucrose but that it holds back all but the finest colloidal particles. Two methods are given for progressively decreasing the size of the pores until the cellophane becomes a very fine molecular sieve. A sieve structure as the chief factor seems most in accordance with our experience of this and other ultrafilters. Collodion membranes may also be used as molecular sieves but their properties are inconstant. Bedicher is a very fine and rapid filtering ultrafilter and pig's bladder holds back a fair proportion of such molecules as sucrose and potassium chloride. Notes are made on the behavior of cellophane in aqueous and non-aqueous solutions. It is emphasized that ultrafiltration is distinctive and has but little relation to diffusion, dialysis, osmosis, electroosmosis or thermodynamics.

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EFFECTS OF CYANIDE ON THE PROTOPLASM OF AMEBA.

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(Accepted for publication, May 30, 1928.)

Within the last 75 years a vast amount of information has been obtained concerning the physiological action of cyanide on organisms. Most of the literature, however, deals primarily with the effects of potassium cyanide on oxidation, while comparatively little information seems to exist in regard to the direct action of cyanide on protoplasm. Since most of our information was obtained from oxidation experiments with potassium cyanide (1, 2, 3), and since it is known that hydrogen cyanide passes through living membranes (4, 5), it was thought desirable to study in some detail the effects of hydrogen cyanide and potassium cyanide on the physico-chemical properties of protoplasm. The work here reported deals with the effects of hydrogen cyanide and potassium cyanide on the viscosity of the protoplasm of a small species of ameba of the *proteus* group.

Method.

Amebæ were immersed in aqueous solutions of HCN or KCN and the same solutions were injected into other individuals by means of Chambers' micromanipulator. The concentrations of the cyanide solutions ranged from $N/10$ to $N/3,000$ and the pH values were changed by the addition of NaOH or HCl. The organisms were studied with transmitted light and with dark-field illumination. It was previously determined that Brownian movement (6) may be used as an index of the comparative viscosity of the protoplasm of amebæ; an increase in the rate of Brownian movement denotes a decrease in the viscosity and a decrease in the movement an increase in viscosity. One criticism of this method is the lack of quantitative results. It has, however, an advantage over other methods in that the viscosity changes in the protoplasm may be noted without interfering with the organism under observation.

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Immersion Experiments.

N/10 HCN: Amebæ immersed in N/10 HCN solutions, with pH values of 4.6, 7.0 and 7.4, show an immediate increase in protoplasmic viscosity which is quickly followed by an internal reorganization of the protoplasm. The granular portion of the protoplasm collects into numerous minute spheres which are suspended in a hyaline matrix (Fig. 1). The spheres are gelled at pH 4.6 while at pH 7.0 and 7.4 the granules within the spheres show slight Brownian movement indicating a gel of low viscosity. The separation of the granular portion of the protoplasm into dense spheres also occurs when amebæ are immersed in a HCl solution with a pH of 4.6. Higher concentrations of HCl (pH 1.6) produce an immediate gelation of the entire protoplasm (6). Thus it appears that the formation of the granular spheres is an intermediate stage between liquefaction, which occurs when amebæ are exposed to dilute cyanide solutions, and gelation of the protoplasm.

N/100 HCN: When amebæ are immersed in N/100 HCN solutions with pH values 5.0, 6.6 or 7.4, the viscosity of the protoplasm is slightly increased for a few seconds, then followed by a swelling of the protoplasm and a marked increase in the rate of Brownian movement. The cell assumes a spherical form and the contractile vacuole enlarges enormously and finally breaks, liberating the enclosed fluid which mixes immediately with the cytoplasm. The cell membrane is either ruptured, allowing the liquid cytoplasm to escape into the surrounding medium or it remains intact. If the cell membrane is not broken, the granular portion of the protoplasm collects into one large sphere which is suspended in a hyaline medium (Fig. 2). The cell membrane eventually dissolves and the cell disintegrates.

N/300 HCN: Amebæ placed in N/300 HCN solutions with pH values of 5.6, 6.6 and 7.4, remain active for a few seconds, then locomotion ceases and the animal retains its life-like position with its pseudopodia extended. This quiescent stage is accompanied by an increase in viscosity of the protoplasm and the animals ultimately swell and assume a spherical form, which is characterized by a marked decrease in viscosity. The contractile vacuole enlarges to many times its original size and several accessory vacuoles appear. The surface

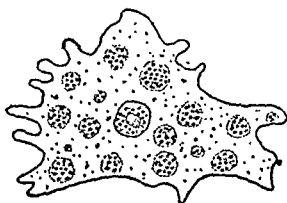


FIG. 1.

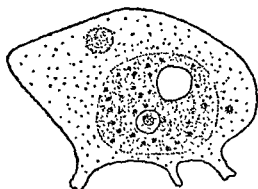


FIG. 2.

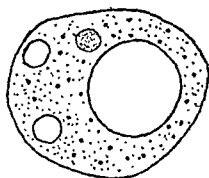


FIG. 3.

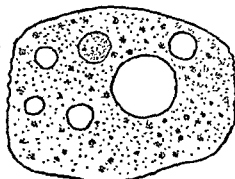


FIG. 4.



FIG. 5.

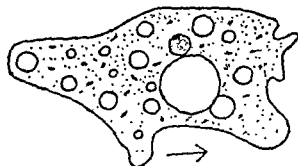


FIG. 6.

FIG. 1. Free-hand sketch of amoeba killed with $N/10$ HCN, showing the formation of minute spheres of protoplasm suspended in a hyaline area.

FIG. 2. Sketch of amoeba killed in $N/100$ HCN. Protoplasm collected in one large sphere.

FIG. 3. Sketch of amoeba immersed in $N/300$ HCN, showing the enlarged contractile vacuole.

FIG. 4. Drawing of amoeba immersed in $N/3000$ HCN for 16 hours.

FIG. 5. Drawing of amoeba immersed in $N/100$ KCN, showing the limax form.

FIG. 6. Sketch of amoeba injected with HCN or KCN. Animal alive and moving in the direction indicated by the arrow.

membrane dissolves after several hours and the cell disintegrates (Fig. 3).

N/3000 HCN: Amebæ immersed in N/3000 HCN solutions with pH values of 6.6 and 7.4, flatten out into a very thin layer on the substratum. This reaction is accompanied by a decrease in protoplasmic viscosity. The contractile vacuole becomes enlarged but does not function; the animals gradually swell and disintegration occurs after 24 to 48 hours (Fig. 4).

N/10 KCN: Amebæ immersed in N/10 KCN solution, pH 9.8, die very quickly; the cell membrane dissolves and the protoplasm mixes with the surrounding solution. The same reaction is obtained when amebæ are placed in NaOH or KOH at a pH of 9.8, thus it seems that the effect of N/10 KCN (pH 9.8) on amebæ is due largely to the alkalinity of the solution.

Amebæ immersed in N/10 KCN solutions with pH values of 6.8 or 7.4 show an initial increase in protoplasmic viscosity, which is followed in 1 to 2 hours by a liquefaction of the protoplasm and disintegration of the cell.

N/100, N/300, N/3000 KCN: Amebæ immersed in N/100, N/300 and N/3000 KCN solutions (pH 6.8 and 7.4) elongate into the limax form and continue locomotion for from 30 minutes to 24 hours depending upon the concentration of the cyanide solution. The animals gradually swell which is immediately followed by liquefaction of the protoplasm and disintegration of the cell occurs within 3 days (Fig. 5).

Injection Experiments.

By means of the Chambers' micromanipulator, amebæ were injected with HCN or KCN. The concentrations ranged from N/10 to N/3000 and the pH values from 4.6 to 7.4. The cyanides at different concentrations and with different pH values produce similar effects on the internal protoplasm of amebæ. The animals after injection become highly vacuolated and assume a fan shape (Fig. 6). The anterior end spreads out into a very thin layer on the cover slip. Brownian movement and streaming of the protoplasm is accelerated; the contractile vacuole enlarges slightly but usually does not contract unless it is lodged in an angle of an advancing pseudopodium or comes

in contact with the cell membrane. The animals recover unless the dosage is so large that it injures the membrane.

Insertion of a needle into the protoplasm of an ameba results in a local gelation at the point of stimulation. As locomotion of the animal continues the gelled area lags behind and eventually constitutes the posterior region of the organism and the ameba assumes a limax form. Ultimately the gelled region is assimilated by the organism and the animal returns to its normal shape.

Injections of distilled water into amebæ result in a dilution of the protoplasm, streaming of the protoplasm is increased and the contractile vacuole contracts at irregular intervals. For a more detailed account of the effect of injections of distilled water on the contractile vacuole the reader is referred to the paper of Howland and Pollack (7).

Tearing the Surface Membrane.

The cell membrane of a normal ameba is elastic and it is possible to stretch it by means of microdissection needles into a long thread-like structure which appears to be fibrous in texture. When released the membrane returns to approximately its former position. With considerable care the animal may be pulled into two parts without any escape of the internal protoplasm and the two sections will continue locomotion. When a small tear is made in the cell membrane, a portion of the internal protoplasm flows from the torn surface and the injury is repaired by the formation of a new membrane. If the tear is sufficiently large, the protoplasm flows out of the cell and mixes with the surrounding water. When amebæ are immersed in aqueous solutions of HCN or KCN and the cell membrane torn, a new membrane is formed over the injured surface, which indicates that the internal protoplasm has not been greatly injured.

Injection Followed by Immersion.

The results of the experiments on immersion, injection and tearing of the cell membrane, seem to indicate that the toxicity of cyanides is due to their action on the cell membrane and that the internal protoplasm is not permanently injured. Since it has been shown that HCN (4, 5) passes through living membranes and that the cyanides decrease

the viscosity of the internal protoplasm of amebæ, it was thought desirable to ascertain whether the toxicity of cyanide is due entirely to its effect on the the cell membrane or to an effect on the membrane and the internal protoplasm. Twenty-five to thirty amebæ in a single experiment were injected with HCN or KCN and then quickly immersed in cyanide solutions. A higher concentration of intracellular cyanide was obtained by this means than would have been obtained from immersion in a cyanide solution. If the toxicity of cyanide is due to an effect on the cell membrane and internal protoplasm, the injected individuals should die in a much shorter time after immersion than the uninjected animals. The results, however, show that the injected organisms do not die any sooner after immersion, than do the uninjected cells immersed in the same cyanide solutions. This seems to indicate that the toxic action of cyanide is exclusively a surface one.

SUMMARY.

The experiments seem to indicate that the toxicity of HCN and KCN for amebæ is due to their effect on the cell membrane and not on the internal protoplasm.

Concentrated solutions (N/10–N/300) of HCN or KCN produce an initial increase in viscosity of the protoplasm of amebæ (immersed) which is followed by liquefaction and disintegration of the cell. Dilute solutions of HCN or KCN decrease the viscosity of the protoplasm of amebæ.

Injections of HCN or KCN into amebæ produce a reversible decrease in viscosity of the protoplasm.

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THE INJECTION OF SULFATES INTO VALONIA.

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(Accepted for publication, May 24, 1928.)

Cells of *Valonia macrophysa*, while ordinarily free from sulfate in the vacuolar sap,¹ occasionally are found in apparently normal state yet giving a test for this ion. In order to determine its effect directly, the following experiments were performed in Bermuda in 1924. The number of cells was small (30) but the results sufficiently definite to justify certain conclusions. The method furthermore is of interest in view of its later employment for the injection of toxic substances.²

Two successive operations were employed. A small amount of sap (about 1/8 of the cell volume) was withdrawn through a fine capillary. This was removed and a second capillary inserted, containing the solution for injection. The contents were forced in under air pressure (taking care that no bubbles entered) until the cell was turgid. The capillary was removed while the pressure was still being applied.

Mortality was high due to the two punctures and the consequent temporary softness of the cell, but a small number of cells survived in both of two groups. In group A a solution of 0.6 M KCl was injected. Of these 2 cells survived and lived 2 weeks, when the experiment was discontinued. In group B a solution of 0.4 M K₂SO₄ was injected, and 3 cells survived. At the end of 2 weeks the sap was removed from all the cells and tested for sulfate ion. Group A gave no test, while group B gave a very strong one (approximately as much as the surrounding sea water). The cells were turgid and seemed normal in every respect. Hence, as far as we can judge from these experi-

¹ Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, v, 225.

² Rapkine and Wurmser have also reported the injection of dyes into the vacuole of *Spirogyra*, using the method of Chambers as modified by the Needhams (Rapkine, L., and Wurmser, R., *Proc. Roy. Soc. London, Series B*, 1927, cii, 128).

ments, the protoplasm can tolerate a considerable amount of sulfate on the vacuolar as well as on the outer surface.

It is also evident that there is no mechanism for rapidly disposing of sulfate after it has entered. If, therefore, the cells have in the past suffered mechanical or other injury which temporarily increased the permeability of sulfates, recovery would still leave a certain amount of these in the vacuole, without subsequent injury to the cell.

SUMMARY.

Potassium chloride and sulfate were injected into the vacuole of *Valonia*. The surviving cells tolerated the presence of these solutions. Sulfate, although ordinarily absent from the sap, is not rapidly eliminated when introduced. Hence the sulfate occasionally found in cells of normal appearance may have entered due to temporary injury followed by recovery.

INTERNAL VERSUS EXTERNAL TOXICITY IN VALONIA.

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(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, June 5, 1928.)

Our bioelectric measurements indicate that in *Nilella* and *Valonia* the protoplasm consists of layers,¹ of which the inner is more sensitive to the action of chloroform than the outer (when both are in contact with the same electrolytes at the same concentration).²

It is a matter of considerable interest to determine the relative sensitivity of these layers by applying a toxic substance to each one separately. The experiments here described were made for this purpose. The cells were divided into two groups. Those of group *A* were allowed to stand in sea water containing a small amount of the toxic agent so that only the outer layer (*X*) was directly exposed. In group *B* sap containing the toxic agent was injected into the cells.³ Here only the inner layer (*Y*) was directly exposed. It seems probable that both *X* and *Y* are relatively impermeable to the toxic agent here employed ($MnCl_2$), at least as long as the protoplasm is not severely injured, and if this is the case we may expect death to occur more rapidly when the poison is applied to the more sensitive layer.⁴

The experiments were carried out in Bermuda on cells of *Valonia macrophysa* (containing from 0.3 to 1.2 cc. of sap) at about 20–25°C.

It was necessary to find a toxic agent which could be measured with a fair degree of accuracy even in very small amounts. Such organic reagents as chloroform or formaldehyde were ruled out because of analytical difficulties. After a number of trials in which Ni^{++} , Cd^{++} , Cu^{++} , Fe^{+++} , Cr^{++} , and Mn^{++} were investigated our choice fell on the last. From the analytical point of view Mn is excellent, since

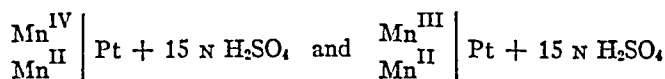
¹ It is assumed that there is an inner layer *Y* and an outer layer *X* both of which are probably non-aqueous: between them there is an aqueous layer *W*.

² Cf. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927–28, xi, 673.

³ Cf. Blinks, L. R., *J. Gen. Physiol.*, 1928–29, xii, 207.

⁴ But if the protoplasm were freely permeable to the toxic agent it would make little difference whether it is injected into the cell or applied from the outside.

it can be determined to 0.01 mg. with good accuracy. It has, however, several valences (2, 3, 4, 6, and 7). In our experiments it was employed in the form of the bivalent salt, MnCl_2 . This may be considered as the salt of a fairly strong base and a strong acid since it undergoes no appreciable hydrolysis. A possible objection is the uncertainty in regard to oxidation. In the sea water the chance of oxidation must be very small. On the other hand we know very little about the oxidation conditions within the vacuole of the cell. We do know, however, that the normal oxidation potentials of the electrodes



are strongly positive: according to Grube and Huberich⁵ over 1.5 plus referred to the hydrogen electrode taken as zero. This indicates such a strong tendency of the higher valence compound to be reduced that it does not seem likely that the cell can oxidize MnCl_2 .

It was hoped at the start that we might be able to determine the maximum concentration of Mn^{++} within the vacuole which the cell would tolerate indefinitely.⁶ This was not realized. A considerable number of cells were found which fulfilled the conditions required, but on analysis they were found to contain so little Mn that the results were very inaccurate. In 17 out of 20 cases where the cells lived for a long time not more than a trace was found. For a similar reason we were unable to determine very accurately the maximum concentration of MnCl_2 in the sea water that the cell would tolerate indefinitely. The plan was therefore adopted of injecting the cells in group A with sap containing MnCl_2 , and allowing them to stand in sea water until dead.⁷ Analyses were then made of the cell contents. In this way data were obtained giving the length of life of cells containing 0.004 to 0.185 per cent of MnCl_2 in their sap. In group B the cells (stabbed with a capillary but not injected with MnCl_2) were simply placed in sea water containing MnCl_2 and allowed to remain until dead.

Technique.

In a previous paper⁵ we have stated that occasionally cells which had been impaled on a capillary and then shaken from the capillary into sea water, healed

⁵ Grube, G., and Huberich, K., *Z. Elektrochem.*, 1923, xxix, 8.

⁶ "Indefinitely" is here used in a limited sense. We know that cells will live for periods of months in sea water in the laboratory, without showing any change in the protoplasm except a slight fading of the color. In this work "indefinitely" simply indicates a period of a month or more during which there is no more change in the appearance than would be displayed by a cell not exposed to Mn.

⁷ The sense in which this word is employed is explained on page 215.

⁸ Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927-28, xi, 202.

up, becoming as turgid as unpunctured cells and continuing to live. This observation formed the basis of our present work. It was found that the proportion of punctured cells which could be made to heal could be very greatly increased by careful handling. Glass capillaries were used, and these were attached to a Luer hypodermic syringe of 2 cc. capacity. The capillaries were considerably finer than those used by us in making the measurements of potential difference. The glass at the tip was extremely thin, and the point was broken off at an angle to facilitate the piercing of the relatively tough cellulose wall. During injection the syringe was filled with sap containing a definite concentration of MnCl_2 . The cell was then grasped firmly between the thumb and forefinger and quickly impaled on the capillary and at the same time a slight pressure was applied to the plunger of the syringe. As soon as the cell was in place on the capillary it was

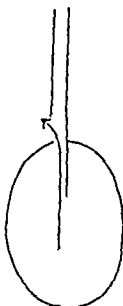


FIG. 1.

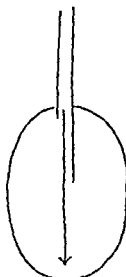


FIG. 2.

FIGS. 1 and 2. Methods of inserting a hollow steel needle into the cell.

relinquished by the fingers, and then a considerable pressure was applied to the piston. At this stage, those cells which showed any sign of leakage around the capillary were rejected. This was done because any severe leak indicated that an undue displacement of the natural sap by artificial sap plus MnCl_2 had probably occurred, and also because when a cell leaked on puncturing there was usually some scouring and tearing of the protoplasm at the site of the leak. This was probably due to the sudden rush of sap out through the minute annular space. Cells so damaged usually failed to heal. The passage of sap + MnCl_2 into the cell could be observed owing to the difference of refractive index of the solutions. It was thus possible to control in a very rough way the amount injected. It is obvious that at the moment of injection the pressure in the vacuole must have been above normal. Nevertheless in only a very few cases (excepting those cells which leaked at the puncture) was there any evidence of rupture of the cell wall.

Further it is of interest to note that those cells which showed any sign of such rupture were invariably large, containing 0.75 cc. or more. The actual transfer of the solution from the syringe to the cell took only a few seconds. While the pressure was still maintained the cell was brushed quickly from the capillary into a large volume of sea water.

This method of injection yielded the greatest number of cells which healed. Deflation of the cell was completely avoided. A second method which was tried and abandoned involved the use of a very fine hollow steel needle. This was ground off at the point at a steep angle. The cell was brought to the needle and forced up on it rather slowly. As Fig. 1 shows there was at the start a passage through which sap could escape as indicated by the arrow. This passage was closed as soon as the impalement was complete (Fig. 2). By applying a slight pressure to the piston the loss of sap was made up by the injected solution. The advantage was that no excess of pressure had to be applied to the interior of the cell, and that after practice it was possible to determine the amount of entering solution quite accurately by controlling the speed at which the hole was closed up. This plan, however, involved some deflation of the cell which is often fatal. If the cell wall is creased or folded the protoplasm is likely to become detached at this point, and this disturbance spreads until death occurs. When the cell is transferred from the capillary to the sea water after injection by the first method described, it is obvious that the moment it leaves the tip there must be a fall in the internal pressure by the escape of liquid through the orifice. Undoubtedly the time of outflow would be very short, both because the internal pressure would be high, and the orifice (which may be regarded as a very short capillary of relatively great diameter) would offer very small resistance to the outflow. Hence we should anticipate a great acceleration of the liquid at the orifice but we should not expect any great scouring of the protoplasm at the circumference for owing to the size of the opening the greatest acceleration would be opposite the center of the orifice, decreasing outward to the circumference. This is in marked contrast to the case previously discussed, where the flow was through a tiny annular orifice. Here owing to the smallness of the opening the greatest acceleration would be opposite the edges. It is probable that most of the material which escaped from the cell when the pressure fell was natural sap; for the injected sap plus MnCl_2 had a greater density and would tend to fall to the bottom of the cell away from the site of the puncture. This could actually be observed in certain preliminary experiments where the pink Co ion was injected. The ideal condition would have been to have released the pressure on the piston at the moment the cell left the capillary. This, however, could not be realized in practice and in consequence the exterior of the cell was subjected to a rain of the toxic solution at this time. However, by holding the cell just above the surface of a large beaker of sea water the length of this exposure was cut down and after injection the cell was removed from the beaker at once and washed in a stream of sea water. This served to rock and rotate the cell, and helped to bring about a thorough mixing of the sap and the slightly heavier toxic solution which was injected. The cell was then

placed in a 50 cc. crystallizing dish containing fresh sea water. This was kept covered to prevent evaporation. Each cell was preserved in a separate crystallizing dish to avoid the effect of exosmosis from other cells.

It was recognized that before healing was complete a certain amount of $MnCl_2$ might escape from the cell by diffusion through the orifice. In order to minimize any effect that this might have on the exterior of the cell, the sea water was changed a few hours after injection and then daily until the cell was healed.

To apply the toxic solution to the exterior the cells were placed in sea water containing varying concentrations of $MnCl_2$, 1 cell to a dish. Weighed amounts of $MnCl_2 \cdot 2H_2O$ were added to sea water, but owing to the uncertain water content, the solutions so made up were analyzed for manganese.

It is plain that when the solution is injected into the cell we must consider not only the action of the toxic solution of the layer *Y'* but also the effect of the puncture on the protoplasm. An attempt was made to eliminate the effect of stabbing by trying to determine the percentage of cells dying as the result of the stab alone but this was abandoned in favor of the plan of impaling all cells. All the cells of group *B* before being placed in the sea water containing $MnCl_2$ were injected with artificial sap containing no Mn. The possibility of the diffusion of $MnCl_2$ through the orifice into such cells must be considered. We might have permitted each cell to heal in sea water before placing it in the toxic sea water. But it was felt that this would give an advantage to these cells in comparison with those of group *A*, which were exposed to the Mn from the moment of injection.

Our experiments were carried out in two series, the following routine being observed. A few cells were injected with sap containing $MnCl_2$, washed, and placed in separate vessels of sea water, then the syringe was washed out with distilled water and finally with artificial sap. Then the same number of cells were injected with artificial sap, washed, and transferred to separate vessels of sea water containing $MnCl_2$. Finally a smaller but proportional number of cells were injected with the sap containing no $MnCl_2$, and these were placed, after washing, in sea water to serve as controls. This process was repeated, until the required number of cells for the series had been obtained. By proceeding in this way we made certain that the cells in each group were injected under exactly comparable conditions.⁹

It was not possible to control, except very roughly, the concentration of $MnCl_2$ applied to the interior of the cell. A small measure of control could be exercised by varying the concentration of the $MnCl_2$ in the injected sap. In practice two solutions were used, containing respectively about 0.05 N and 0.25 N $MnCl_2$. These concentrations represented a compromise. When a solution 0.01 N was injected into 25 cells 18 of them lived for more than 2 weeks without sign of injury. At the end of this period the experiment was terminated. On analysis only 6

⁹ The needles were of course very fragile and when one broke during the stabbing the cell was rejected.

cells showed more than a trace of MnCl_2 present, while in two cases no Mn was detected. On the other hand, when a solution containing 0.1 N MnCl_2 was injected into a group of 9 cells, 8 of them failed to heal and within 48 hours the protoplasm had detached itself from the cellulose wall.

During the first 2 days after injection all the cells were examined carefully for signs of healing. In this respect the behavior of the control cells was of great interest. Many of these showed definite signs of recovery in 6 hours, and in nearly all cases healing was complete (as far as the eye could detect) within 24 to 48 hours. The visible signs of healing are the deposit of a black material in the puncture and the regaining of full turgidity.¹⁰ Most of these cells continued to live indefinitely. A group of such healed cells compared with a group of unpunctured cells kept under similar conditions failed to show an appreciably higher mortality in 20 days. The effect of impalement is apparently shown at once, and if impaled cells heal they will survive almost as well as unpunctured cells. This conclusion is in line with our previous experiments⁸ on potential difference in which cells were impaled and left on capillaries in sea water for periods up to a month, without suffering any apparent injury.

We believe that our procedure eliminated the effect of the impalement. All cells which persistently remained soft were rejected, and all cells which showed complete or even partial healing were kept (by partial healing we mean that a few cells showed the deposition of the black material in the puncture but died before they had completely recovered their turgidity).

It was observed that in the presence of Mn a series of changes took place with considerable regularity. Sometime after the sealing of the puncture, the protoplasm showed long thin areas of a lighter color. These widened, and became still lighter or even colorless. This process continued until the protoplasm was without color except for very small dark irregular patches. During these changes the cell retained its usual turgidity. At length it softened abruptly. At this stage the protoplasm either detached itself spontaneously from the cellulose wall or the detachment occurred when the cell was gently rolled between the fingers. These changes took place in both group A and group B when the concentration of the MnCl_2 was high. At lower concentrations the appearance of the lighter areas was either very much delayed or if they appeared early they spread comparatively slowly. In these cases the protoplasm usually detached itself spontaneously and the "colorless" and "soft" stages were absent. Variations of these main types of behavior were also observed. It was therefore necessary to select a stage in the protoplasmic changes to serve as a reference point in comparing the length of

¹⁰ About 7 per cent failed to heal. These remained soft and within 24 to 72 hours showed a definite detachment of the protoplasm from the cellulose wall. In many of these cases the protoplasmic structure was greatly altered, and in others on gentle rolling between thumb and forefinger the protoplasmic layer broke up.

life of the cells. Only two points seemed suitable, namely: the first appearance of the light streaks, and the detachment of the protoplasm. The first of these was less desirable for it was not always possible to distinguish slight irregularities present at the start from the incipient streakiness. The detachment of the protoplasm was therefore selected as our reference point. There was no detectable diffusion of Mn^{++} or SO_4^{--} into the cell (and no diffusion of Mn^{++} from the cell) at any stage preceding the detachment of the protoplasm. A few hours after this had occurred, however, sufficient diffusion of these ions across the membrane had taken place to be readily detected. It would therefore seem as though this is the point at which the protoplasm becomes freely permeable. In the discussion which follows we shall designate cells which have reached this point as "dead."¹¹

Analyses.

It was not, of course, possible to tell the exact moment when the protoplasm became freely permeable in cases where its detachment was spontaneous and hence in cells of group *A* it was necessary to take into account $MnCl_2$ which might have passed out into the sea water by diffusion. In making the analyses, therefore, the sea water was tested for Mn with the delicate reagent potassium periodate. When Mn was detected, care was taken to extract all the sap from the cell. This was done by piercing it with a fairly fine capillary pipette and by applying considerable suction. The external pressure caused the cell to collapse completely, and drove not only all the sap but also the detached protoplasm into the pipette. The sap was blown from the pipette into a small glass-stoppered bottle and weighed. A certain amount of inaccuracy was therefore introduced into the analysis since the weight would be too great by the weight of the protoplasm. On the other hand since some of the Mn had passed out into the sea water the weight of sap from this cause was too little. We cannot accurately estimate these errors which tend to compensate each other, nevertheless it does not seem that they could have affected the final results very seriously. After the sap had been weighed it was added to the sea water in which the cell had stood and the solution was evaporated with 0.5 cc. of concentrated HNO_3 , to oxidize the protoplasm, and 0.5 cc. of concentrated H_2SO_4 , to change all the chlorides to sulfates. As an extra precaution, because of the large amount of chloride present, a second evaporation with H_2SO_4 was performed. The Mn was then oxidized according to the well known bismuthate method, and compared with standards containing known amounts of Mn.¹² In the group *B* analyses, where no appreciable amounts of Mn were detected in the sea water bathing the cells, the sea water was not included for the analysis, and since it was not necessary to empty the cell com-

¹¹ See also page 210.

¹² For details of the analysis see Standard methods for the examination of water and sewage, American Public Health Association, Boston, 5th edition, 51.

pletely, sap free from protoplasm was obtained for analysis. We have not been able to detect in these two sets of group *B* results any systematic variation comparable with the natural variation of the cells themselves.

DISCUSSION.

The effect of the treatment is shown in Fig. 3. For group *A* (Mn applied externally) the figures were obtained by averaging the length of life of all the cells (usually 18) at each concentration. For

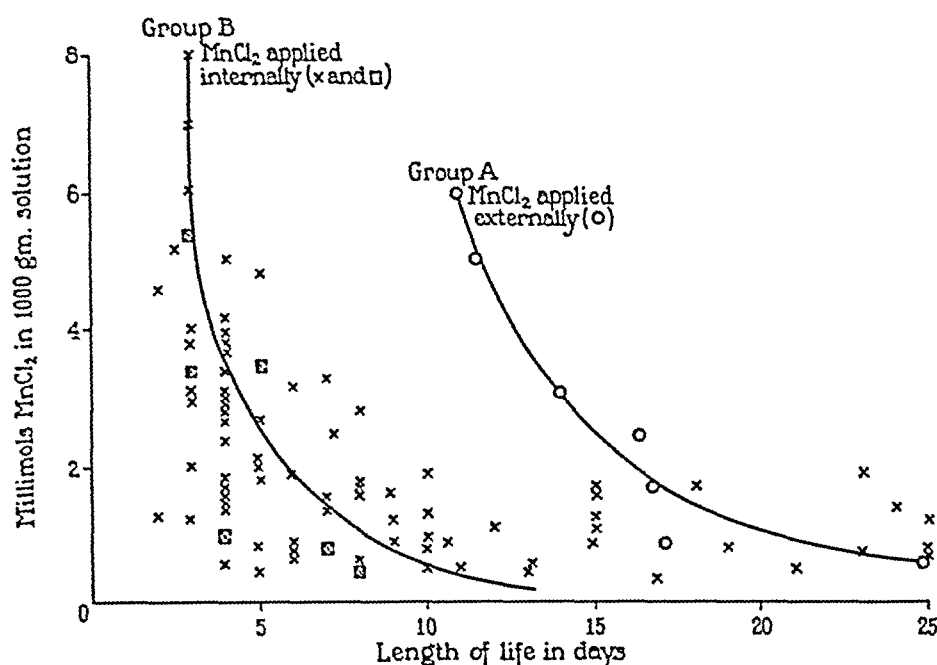


FIG. 3. Duration of life. The points for group *A* are obtained by averaging the length of life at each concentration. Those for group *B* are separate determinations of individual cells, the curve being drawn free-hand without attempting anything more than a rough fit.

group *B* (Mn applied internally) the length of life of each cell was plotted and a curve drawn free-hand without any attempt to do more than make a rough fit. The scattering of the points at low concentrations is to be expected since in general the application of dilute solutions of toxic substances gives very irregular results. (In general the controls lived more than a month after being stabbed.)

It is obvious that the cells of group *A* lived about twice as long as

those of group B. The difference might have been a little greater if the amount of Mn available for combination with the protoplasm had been the same in both cases; but where the Mn was applied externally at the same concentration as in the sap the external volume of solution was greater (10 to 15 cc. for each cell). Hence if the protoplasm combined with most of the Mn there would be more available in the latter case but it is not probable that more than a small fraction of the Mn was taken up by the protoplasm.¹³

Earlier in this paper we spoke of the possibility of the diffusion of MnCl_2 from the sea water into the cell before healing was complete. This might tend to reduce the average life of the cells by subjecting Y as well as X to the action of the toxic agent. This would tend to bring the curves closer together. Hence any error involved would not invalidate the conclusion that the injection of the MnCl_2 produces death more quickly than application to the exterior.

Hydrolysis¹⁴ of MnCl_2 might affect the results by increasing the acidity but our tests indicate that this effect is negligible.

If we take the curves as drawn we see that, for example, when 0.0003 M MnCl_2 is applied internally the cells live about 12 days; to shorten the life of the cell to the same extent by an external application would require about 0.0045 M, a concentration more than 10 times as great.

The simplest interpretation might be to regard the inner layer, Y , as more sensitive than the outer, X , and to suppose that as soon as the inner layer is sufficiently altered to become permeable to MnCl_2 the latter diffuses through Y and attacks X . An attack from the outside would be slower because of the greater resistance of X .

An alternative assumption might be that X is permeable to the toxic agent and Y is not, and that the layer which is permeable is not

¹³ It is of course possible that some Mn may combine with the cell wall in such a way as to be removed from the sphere of action but it does not seem probable that this is a factor of importance, especially as each cell was placed in a separate dish containing 10 to 15 cc. of solution.

¹⁴ The hydrolysis of MnCl_2 appears to have been measured only once. Thus Kullgren (*Z. physik. Chem.*, 1913, lxxxv, 473; see Landolt-Börnstein, *Physikalisch-chemische Tabellen*, Berlin, 5th edition, 1923, ii, 1170) found by the sugar inversion method that a 0.25 M MnCl_2 solution at 100°C. was hydrolyzed only 0.0017 per cent.

injured by the $MnCl_2$ which diffuses through it so that when the solution is injected Y will be attacked at once, but X will not be injured until after the alteration of Y .¹⁵ When the toxic solution is applied to the outside of the cell Y will be subject to attack only after the toxic substance has diffused through X .

Even if there are differences of permeability it is probable that cells also have differences of sensitivity as well to deal with. The assumption that X and Y are unlike is in harmony with our previous work on potential differences across the protoplasm of *Valonia*^{16,17} and with the investigations of de Vries¹⁸ on the marine alga *Griffithsia*¹⁸ and with the investigations of Höber¹⁹ in so far as they may be interpreted to mean that the inner and outer surfaces do not act alike. But the statement of Höber²⁰ that the inner and outer surfaces of the protoplasm are unlike seems to apply rather to a difference between the inner and outer surfaces of X .

It is probably not worth while to attempt to compare our results with those of Chambers and his coworkers who have in some cases found much less toxicity when a substance is injected into X than when applied to the outside,²¹ since in these cases the substance was not injected into a pre-existing vacuole as in the case of *Valonia* where the mechanism involved may be quite different.

¹⁵ Throughout this part of the discussion the destruction of either layer is considered to be equivalent to death.

¹⁶ Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.* 1927-28, xi, 193.

¹⁷ Experimental evidence seems to indicate that X is actually permeable to certain ions, while Y may not be.

¹⁸ Osterhout, W. J. V., *Science*, 1913, xxxviii, 408.

¹⁹ de Vries, H., *Jahrb. wissenschaft. Bot.*, 1885, xvi, 465. See also Kützing, *Ber. bot. Ges.*, 1909, xxvii, 589; *Arch. Entwicklungsmech. Organ.*, 1910, xxx, 351 (Festschrift für Wilhelm Roux); *Z. Bot.*, 1910, ii, 689.

²⁰ Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, Leipzig, 1926, 732.

²¹ A particularly striking case is that of picric acid, as described by Pollack and Howland (Pollack, H., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 145. Höber, R. B., and Pollack, H., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 221).

SUMMARY.

When $MnCl_2$ is injected into the cells of *Valonia macrophysa* they live only about half as long as when the same concentration is applied to the exterior of the cell. This is due to toxic action and not to the mechanical disturbance accompanying the injection (since all cells were stabbed in the same manner by the capillary).

A variety of explanations are suggested, all of which involve a difference between the inner and outer layers of the protoplasm.

STUDIES ON PERMEABILITY OF MEMBRANES.

VI. MENSURATION OF THE DRIED COLLODION MEMBRANE (CALCULATION OF DIMENSIONS AND OF RELATIONS TO CERTAIN BIOLOGICAL MEMBRANES).

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In a previous paper of this series a flat form of the dried collodion membrane and its method of preparation was described (1). As this form of membrane presented certain advantages in durability and convenience of manipulation over the previously used bag membranes, it was the type used in most of the subsequent investigations. Electric transfer experiments in which relatively strong electric currents were used (2, 3) and diffusion experiments continued over a period of several months (4) were both made possible because of the resistant and stable properties of these membranes. In experiments to be reported on in the near future the same type of membrane was used for conductivity measurements. In view of the many experiments made with these membranes it has seemed desirable to publish a brief account of measurements of the dimensions and physical properties of some of them. Such measurements are necessary when one wishes to compare diffusion rates, conductivity values, etc., with results obtained with other types of membranes. In the last paper of this series (4), dealing with the diffusion of non-electrolytes, the rates of diffusion of acetone, glycerol, urea and glucose were measured for a number of these membranes but as the result desired was a ratio of the rates at which different substances diffused it was not necessary to reduce the results with any one membrane to terms of unit area and thickness. Nevertheless some points of interest can be brought out by so doing as will be shown presently.

The membranes used in the present study were selected from those

previously used in the non-electrolyte diffusion experiments. The following measurements were made:

1. *Area*.—The membrane was removed from its bell-shaped glass supporting frame by cutting the attachment around the outside rim with a knife. With scissors the membrane was then trimmed in such a way as to remove as nearly as possible those bits of collodion whose only purpose was to glue the membrane to its frame. There remained a smooth, flat and approximately circular piece of collodion comprising at least most of the previously functioning surface. This was placed in water. For an estimation of the functioning area of the membrane it would have been sufficiently accurate to consider the membrane as a perfect circle and calculate the area from measurements of the diameter. However, as it was desired to use the exact area of the piece removed in calculating the membrane thickness, another method of obtaining the area was employed. A piece of good grade rather heavy white paper was selected and from it a square exactly 15 cm. on each side was cut. The weight of this piece of paper was determined. The membrane was then quickly blotted, placed on a piece of glass with the paper over it and the outline traced with a pencil. The tracing was repeated so that four outlines were made on the one piece of paper. These were now carefully cut out and weighed. The area of the piece of membrane was then calculated from the proportion.

$$\text{Membrane area: } 225 \text{ cm}^2. = \frac{\text{Weight of 4 tracings}}{4} : \text{Weight of } 225 \text{ cm}^2.$$

2. *Weight of Wet Membrane*.—By the weight of a wet membrane is meant the total weight of a membrane whose pore channels are still filled with water. Following the method of Hitchcock (5) each membrane was quickly blotted between filter papers, wiped dry of surface water, placed in a covered moisture dish and weighed. The average of several such weighings was taken as the correct weight. At first glance it would seem that such weighings must be extremely inaccurate because varying amounts of water might be removed from the pores by the filter paper. It is of interest, then, that the average deviation from the mean rate was never greater than 0.36 per cent and for all the membranes studied averaged 0.12 per cent.

3. *Weight of Membrane Immersed in Water*.—Several weighings were made of the membrane when it was suspended in water by a fine hair, the average figure being entered as the correct weight. The temperature of the water was recorded.

4. *Weight of Dry Membrane*.—After all other measurements had been completed the membrane was dried to constant weight in an oven at 60°C. This usually was reached in about 6 hours. With the type of collodion used it was not possible to dry at 100°C. as did Hitchcock. This temperature caused the collodion to become yellow and to lose weight steadily.

From the above measurements the following membrane dimensions were computed:

(a) Thickness: The weight of the wet membrane in air minus the weight when immersed in water equaled the displacement of water. The latter figure when corrected for temperature gave the volume of the membrane. The volume divided by the area gave the thickness. The average thickness of all the membranes studied was 0.0903 mm. This method of obtaining an accurate estimate of the thickness of a thin membrane is essentially the same as that used by Bjerrum and Manegold (6). These authors did not determine the weight of their membranes suspended in water but by using in their calculations a fixed value for the density of collodion reached the same result.

(b) Average cross-section area of pores: On the assumption that the weight of the wet membrane is merely equal to the weight of the dry membrane plus the weight of the water within the channels the latter weight was found and from it by temperature correction (in this case of negligible importance) the volume of the pores was computed. The pore volume divided by the membrane thickness gave an estimate of the average pore area on cross-section. Whether the channels are sufficiently uniform in calibre to allow this figure to be regarded as the area at any cross-section, and more especially the pore area at the two surfaces, it is of course not possible to say.

(c) Part of membrane occupied by pores: The proportion of the membrane occupied by pores was determined by finding the ratio between the pore volume and the volume of the whole membrane. The same percentage result could also be obtained by finding the ratio between the pore area and the area of the whole membrane. For the membranes studied it was found that the pores occupied from 10.5 per cent to 15.4 per cent of the entire membrane (average 13.1 per cent). In studying a series of collodion membranes of varying permeability but of the usual type used for dialysis Hitchcock (5) found that the more permeable membranes of his series were composed of 7 to 8 times as much water as collodion while the less permeable contained half as much water as collodion. In this respect then the figures given here represent a continuation of Hitchcock's series, the dried type of membrane being much less permeable and containing a much smaller proportion of water.

(d) Density of the wet membrane: The density of the wet membrane was determined by dividing the weight of the wet membrane by

its volume (loss of weight in water corrected for temperature). This figure represents the density of the membrane as a whole and in the form that it has been used in our experiments. It is of course a component result depending on the density of dry collodion and of water and of the proportions of the two in the membrane. In our series it varied from 1.567 to 1.602 and averaged 1.583.

(c) Density of the dry membrane: This figure represents merely the density of the dried collodion from which the membranes were made. As the data already collected permitted its estimation for each membrane, it was determined in each case, the result being a check on the accuracy of the original measurements. The weight of the membrane immersed in water subtracted from the weight of the membrane after drying gave (after the usual temperature correction) the volume of the collodion framework of the membrane exclusive of the pore volume. The dry weight divided by this volume gave the density. In the present series the variations were from 1.669 to 1.675, the average density being 1.672. The average deviation from the mean was only 0.11 per cent. The figure is in satisfactory agreement with that of 1.653 given by Hitchcock (5) for the collodion used in his experiments and of 1.72 for that used by Bjerrum and Manegold (6).

DISCUSSION.

The various measurements enumerated in the foregoing paragraphs have been tabulated in Table I. Although we have given these measurements principally to place on record the dimensions of the membranes used in our various experiments, still a few items of interest may be brought out in connection with them.

In the last paper of this series in publishing the results of diffusion experiments the amounts of acetone, urea, glycerol and glucose passing the membranes were compared. The results for each membrane were not expressed in terms of unit thickness and area as only the ratio between the rates holding for two different substances was being studied. In explaining the large differences in the diffusion rates between the substances of larger and smaller molecular size, it was supposed that the rate of diffusion of each substance was proportional to a certain "available pore area." The available pore area differed from the total pore area by the amount of pore area distributed among

holes too small to permit the passage of that substance. Glucose diffused more slowly than glycerol and glycerol many times more

TABLE I.

Membrane	Area	Thickness	Density wet	Density dry	Total area of pores at any cross-section	Part of membrane occupied by pores
	cm. ²	mm.			cm. ²	per cent
F-1	29.491	0.1056	1.572	1.673	4.401	14.9
F-2	28.930	0.1185	1.584	1.670	3.720	12.9
F-3	31.112	0.0894	1.581	1.670	4.113	13.2
F-5	32.847	0.0950	1.594	1.674	3.880	11.8
F-7	30.043	0.1036	1.589	1.672	3.704	12.3
F-8	27.871	0.0681	1.602	1.674	2.934	10.5
F-9	29.779	0.0886	1.567	1.669	4.486	15.1
F-10	30.275	0.0816	1.593	1.673	3.607	11.9
F-11	26.462	0.1044	1.591	1.671	3.120	11.8
C-6	30.232	0.0766	1.570	1.675	4.655	15.4
C-7	27.514	0.0615	1.571	1.669	4.027	14.6

TABLE II.

Membrane No.	Diffusion of glycerol			Diffusion of acetone		
	Rate for entire membrane	Rate for unit portion of membrane	Average deviation from mean unit rate	Rate for entire membrane	Rate for unit portion of membrane	Average deviation from mean unit rate
			per cent			per cent
F-1	1.71	0.410		123.0	29.48	
F-2	1.50	0.477		83.2	26.47	
F-3	1.56	0.339	43.6	125.3	27.20	13.3
F-5	0.78	0.191		76.8	18.79	
C-6	7.04	1.157		167.4	27.52	
C-7	2.84	0.433		243.5	37.13	

In the first column for each substance is given the diffusion rate for each of the membranes as previously determined. In the second column this rate has been standardized by multiplying by the membrane thickness (expressed in units of 0.1 mm.) and dividing by the cross-section pore area. It will be seen that the standardized rates are considerably more uniform in the case of acetone than with glycerol.

slowly than acetone because a much larger percentage of the total pore area was available for the substances of smaller molecular size.

In other words it can be inferred from this theory that as the molecular size of the diffusing substance decreases the value of the available pore area will approach that of the total pore area. It would then seem that in studying the diffusion rate of substances of small molecular size one might hope to find some relation to the total pore area whereas with substances of larger molecular size no such relation should exist. The experiments with acetone and glycerol seemed best suited for testing this assumption. Eight membranes were used in these experiments; six were measured by the method described in this paper. In making this test the values for the diffusion rates of acetone and glycerol given in the last paper (4) (Table IV) were reduced to terms of unit thickness (0.1 mm.) and unit pore area (1.0 sq. cm.) by using the dimensions given in Table I of this paper. The results are listed in Table II. It will be seen that according to expectation the standardized rates for acetone are much more uniform than those for glycerol. With glycerol the average deviation from the mean result is 43.6 per cent while with acetone it is only 13.3 per cent. Better agreement than this could scarcely be expected for it must be remembered that according to the theory some part of the total pore area must be taken up by pores too small to permit the passage even of acetone.

It has already been mentioned that one purpose in recording these measurements is to make possible comparisons with other types of membranes and especially with biological membranes. Such comparisons are desirable and even necessary to determine within what limits the dried collodion membrane may be taken as a model for very much more complicated cell membranes. Unfortunately peculiar obstacles stand in the way of him who would determine the dimensions of cell membranes. These membranes are extremely thin and delicate and any kind of direct measurements are impossible. Within recent years several investigators, notably Fricke (7) and McClendon (8), have described a method of approaching the problem indirectly. Both of these investigators have estimated the thickness of the red blood cell membrane from measurements of the electric capacity made with high frequency currents. At best the values given must be regarded as approximations only, for even if the factor of experimental error be eliminated they depend upon assumed values

for the dielectric constant of the membranes. Nevertheless, the method is extremely ingenious and is the best approach to a difficult problem that has been made so far. McClendon has calculated the cell membrane thickness as 3×10^{-8} cm. if he assumes a dielectric constant of 3 and as 3×10^{-7} cm. if he takes the dielectric constant as 10. Fricke, assuming a dielectric constant of 3, but with capacity measurements somewhat different from those of McClendon, calculated the thickness as 3.3×10^{-7} cm. In other words the membrane thickness is almost within the range of molecular dimensions. Recognizing the approximate nature of these figures it is interesting to use them for making some rough comparisons between the dried collodion membrane and the membrane of the red blood cell.

In previous papers there have been enumerated some of the potential effects observable when the dried collodion membrane represents the interphase in concentration chains and in chemical chains. It will be recalled that these potential differences do not depend on the thickness of the interphase but merely on the nature and concentration of the adjacent solutions and on certain properties of the membrane itself. On the other hand the force acting on any charged particle within the membrane depends on the intensity of the electric field at this point and the field intensity does depend on thickness. The electric field intensity (ϵ) is given by the formula

$$\epsilon = \frac{E}{d}$$

where E is the potential difference between the two borders of the membrane and d the membrane thickness. It is thus evident that, for a given potential difference, the thinner the membrane the greater the intensity of the electric field and consequently the greater the force acting upon a charged ion within its borders. If we take the thickness of the average dried collodion membrane of the type used in these experiments as 1×10^{-2} cm. and that of the red blood cell membrane as 3×10^{-7} cm. it is evident that equal potential effects will in the case of the cell membrane give rise to an electric field with an intensity 30,000 times greater than in the case of the collodion membrane. In other words very small electromotive effects may have a large significance with biological membranes.

In previously reported experiments in which the transfer number of chlorine in several chloride solutions in different concentrations was determined by means of direct electric transfer experiments it was shown that the electric current itself was capable of altering appreciably the transfer number that existed at the moment of first application of the current. Even with currents of 2 to 4 milliamperes, which were used in most of the experiments, the results of electric transfer experiments were in certain ranges of concentration quite measurably different from transfer numbers estimated for the same membrane from concentration chains. Inasmuch as the rate at which a given electrolyte can diffuse across a membrane depends upon the transfer numbers of its ions (being a maximum when they are each equal to 0.5) it follows that an applied electromotive force can materially change the rate of diffusion of a substance dissociated into ions. For example with one membrane in a medium concentration range with KCl an applied electromotive force of 1 volt was necessary to establish a current of 4 milliamperes across the membrane. Supposing that this membrane was roughly 0.1 mm. thick, this is equivalent to an electric field intensity of 100 volts per cm. To establish the same electric field intensity across the membrane of the red blood cell (thickness taken as 3×10^{-7} cm.) would require a potential difference of only 0.03 millivolt. Potential differences of this magnitude and greater must frequently arise in the animal organism. In fact E. J. Warburg (9) has estimated that a difference in pH of from 7.2 to 7.4 between cells and plasma may give rise to a potential difference of 2 millivolts. It is possible though of course only a surmise that such electromotive effects arising as a result of nerve impulses or because of changes in the concentration of the tissue fluids may be of paramount importance in controlling rates of excretion and secretion by certain cells and be intimately associated with such complex phenomena as the chloride shift.

From the standpoint of the diffusion of non-electrolytes it is interesting to see in how far the dried collodion membrane might serve as a model of a cell membrane if its dimensions were similar. At first glance the recorded rates for the diffusion of glucose with these collodion membranes seem extremely slow and this slowness seems at variance with the rapidity with which the amount of glucose on the

two sides of the red blood cell membrane may become equalized. It will be recalled that with the collodion membranes the fastest rate recorded was only 1.57×10^{-3} millimols per hour for the whole membrane with a diffusion pressure of 2.4 atmospheres. This amounts to 1.56×10^{-3} mg. per minute per sq. cm. of membrane surface at the same pressure. The thickness of this membrane was 7.7×10^{-3} cm. Now it has been estimated that in 1 cc. of blood there are 5,000 sq. cm. of membrane surface (8). If we again take the thickness as 3×10^{-7} cm. and suppose that some physiological event has raised the plasma glucose 72 mg. per 100 cc. above the cell glucose (producing a diffusion pressure of 0.1 atmosphere at 37 C.) we can calculate that if the substance of the red blood cell membrane were dried collodion glucose would diffuse across the membrane at a rate of 8.5 mg. per minute per cc. of blood as long as the pressure was maintained. That is, the entire 72 mg. per 100 cc. originally present as an excess in the plasma could have diffused across the cell membranes in about 5 seconds. Of course the pressure is not maintained for as diffusion continues the pressure falls. But at least we can say that within several minutes the difference in concentration between cells and plasma would no longer be detectable and this is quite in accord with what little is known concerning the rapidity with which glucose may be exchanged between cells and plasma across the normal red blood cell membrane.

SUMMARY.

The flat type of dried collodion membrane used by Michaelis and his associates in numerous investigations has been subjected to mensuration in order that the dimensions of these membranes may be placed on record. The membranes had a functioning area of about 30 cm., were approximately 0.1 mm. in thickness and were composed on the average of 87 per cent by volume of collodion and 13 per cent by volume of pores.

In reviewing some of the previously reported results of diffusion experiments with non-electrolytes in the light of the calculated values for the total pore area for the same membranes additional evidence was presented to show that a smaller molecule (acetone) probably utilizes a much larger percentage of the total pore area for its diffusion than is available for a larger molecule (glycerol).

By using the figures of Fricke and McClendon for the thickness of the membrane of the red blood cell some comparisons were drawn between the dried collodion membrane as a model for certain biological membranes and the red blood cell membrane. In these comparisons emphasis was placed on the exaggerated importance of small electromotive forces and very slight permeabilities when these were associated with membranes of such extreme thinness as the red blood cell membrane.

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9. Warburg, E. J., *Biochem. J.*, 1922, xvi, 329.

THE PREARGININE IN EDESTIN AND ITS RESISTANCE TO HYDROLYSIS.

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I.

INTRODUCTION.

In a previous paper¹ it was shown that the amount of arginine occurring as such ($pK' = 8.1$) in proteins is a small portion of the arginine found on hydrolysis. The remainder is produced by the hydrolysis of a weak basic group ($pK' = 4.6$) to which the name "prearginine" is given.²

II.

Edestin.

We now turn to edestin (a protein from hemp) which gives a high yield in arginine on hydrolysis. The available data on edestin in alkaline solution are inconsistent and we obtained new titration data in this range. Since our new data are no marked improvement over the old we will not publish them but present our analysis of the data assembled by Cohn.³ Curve *A* in Fig. 1 shows the experimental values. The dotted portion of Curve *A* (in alkaline solution) indicates that the protein is modified in that range. The true curve in alkaline solution should agree with Curve *B*₃.

Curve *B*₁ corresponds to the amino acids found on hydrolysis. In

¹ Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 629.

² The name "prearginine" is given to that portion of the protein molecule having a basic group ionizing about pH 4.6 and yielding arginine on hydrolysis. It may be composed of one, or of more than one, amino acid. Such a weak basic group should consist of a primary amino group attached to a conjugated unsaturated system (perhaps cyclic) as in aniline or cytosine.

³ Cohn, E., *Physiol. Rev.*, 1925, v, 349.

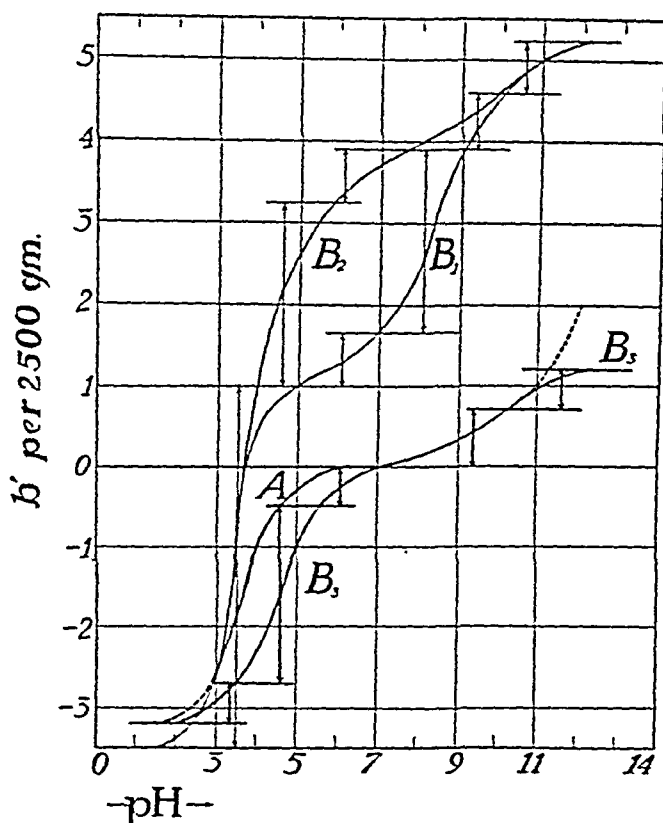


FIG. 1. A. Experimental titration curve of edestin. B_1 , B_2 , B_3 , curves calculated to correspond with various amounts of the ionizable groups.

TABLE I.

Analysis of Edestin Data Demonstrating the Relation between Arginine and Prearginine.

Groups	Sources	Indices in edestin†	Equivalents per 2,500 gm.		
			On hydrolysis	Titration data	Difference
Acidic	Dicarboxylic acids*	$pG_1' = 3.0†$	4.5*	0.5	-4.0
	Tyrosine	$pG_8' = 9.4$	0.7	0.7	0
Basic	Prearginine	$pG_2' = 3.6†$	0	2.2	+2.2
	Histidine	$pG_3' = 5.1†$	0.6	0.5	-0.1
	Arginine	$pG_4' = 8.1$	2.2	0	-2.2
	Lysine	$pG_5' = 10.6$	0.6	0.5	-0.1

* The value for the free carboxyl groups is the total dicarboxylic acids minus the amount bound as amides ($7.8 - 3.3 = 4.5$ equivalents).

† The indices of carboxyl groups, of prearginine and of histidine in *gelatin* are about 3.5, 4.6 and 6.1 respectively.

B_2 there is no arginine but an equal amount (2.2 equivalents) of pre-arginine. B_3 represents the same groups but in the amounts given in Table I (under "titration data"). This last curve (B_3) is probably correct on the alkaline side but must be shifted to the left on the acid side in order to agree with the experimental data (Curve A). This indicates that the COOH index is a little lower than 3.5 (say 3.0) and the prearginine and histidine indices are lower than their usual values (about 3.6 and 5.1).

The results of the titration curve analysis are given in the next to the last column of Table I. The preceding column gives the equivalents of the amino acids found on hydrolysis. The values for histidine and lysine (0.6 and 0.6) are taken from Van Slyke⁴ rather than from Vickery and Leavenworth⁵ since the latter values (0.3 and 0.4) are lower than those we obtain from titration data (0.5 and 0.5).

It will be observed that there is *no free arginine group in edestin* and that all the 2.2 equivalents found on hydrolysis *exist in this protein as prearginine*. 4.0 equivalents of the "free" carboxyl groups do not ionize (perhaps bound as anhydride). All the other groups *i.e.*, tyrosine, histidine and lysine exist in edestin in approximately the amounts found on hydrolysis.

III.

Hydrolyzed Edestin.

We made up four samples of edestin and hydrolyzed them with pepsin in acid solution (data given in Tables II to V). The hydrolyses were stopped at different points, neutralized with an amount of alkali exactly equal to the acid and titrated electrometrically.

Edestin contains 18.6 per cent nitrogen, or 33.3 mols per 2,500 gm. 9.0 equivalents of this is non- α -nitrogen in the basic groups, leaving 24.3 equivalents of α -nitrogen. The initial acid-combining capacity (due to the ionizable non- α -nitrogen) is 3.2 equivalents. Hence the degree of hydrolysis given by the equation

$$\text{Per cent hydrolysis} = \frac{100 (A - 3.2)}{24.3}$$

⁴ Van Slyke, D. D., *J. Biol. Chem.*, 1911, x, 15.

⁵ Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, 1928, lxxvi, 707.

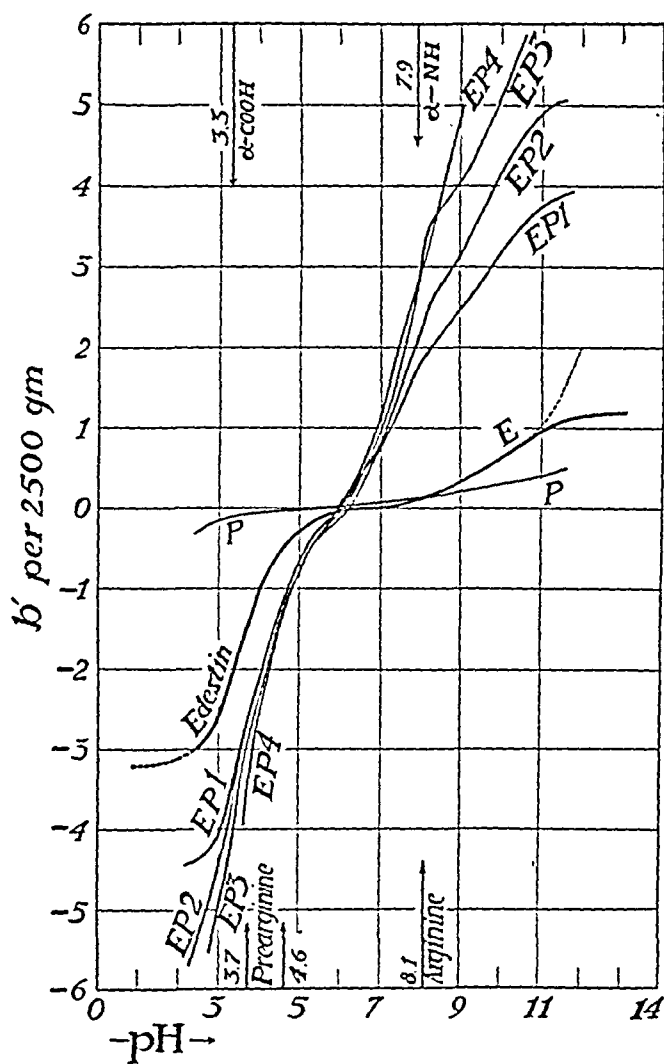


FIG. 2. Titration curves of edestin (E), of pepsin (P) and of solutions of edestin hydrolyzed to different degrees with pepsin (EP_1 , EP_2 , EP_3 , and EP_4).

where A is the acid-combining capacity (in equivalents per 2,500 gm.) of a hydrolyzed solution. On this basis the four samples of edestin were hydrolyzed 5 per cent, 14 per cent, 18 per cent and about 30 per cent respectively. These were titrated and the data are given in Tables II to V.

None of these solutions showed any change of prearginine into arginine in their titration data (given in Fig. 2). Such a change would be manifested by a drop in the EP curves at 3.7 (prearginine) and a rise at 8.1 (arginine), as compared with the Curve E of edestin. Comparison curves⁶ were drawn and in the first three samples corroborated this conclusion (that prearginine is not converted into arginine in these solutions). However the comparison curve of the fourth sample showed that it had hydrolyzed so far that the buffer effect of the α carboxyl groups (3.3) and the α amino groups (7.9) would make it impossible to observe this change if it occurred.

Thus we know that prearginine is not destroyed by hydrolysis up to 18 per cent with pepsin. We also know that complete hydrolysis destroys it, since arginine can be isolated quantitatively from the resulting solution.⁵

Hunter⁷ has shown that the rate of hydrolysis of edestin with trypsin indicates that arginine exists in two forms. We would suppose that the more resistant form is prearginine except that the data (Table II) indicate, in this particular protein, that there is no free arginine group (all existing as prearginine). Felix⁸ showed that part of the "arginine" from hydrolyzed proteins was not precipitated in the usual manner. This fraction may be prearginine. Edlbacher⁹ separated fractions high in arginine from oxidized proteins. Part of these arginine-rich fractions resisted further hydrolysis. The significance is doubtful.

⁶ Simms, H. S., and Levene, P. A., *J. Biol. Chem.*, 1926, lxx, 319. The curves in Fig. 2 would be more accurate if the curve for the corresponding amount of pepsin were subtracted from the observed curve, but this would not affect the conclusions. Titration data of pepsin are given in Table VI.

⁷ Hunter, A., *Trans. Roy. Soc. Canada*, 1925, xix, 1.

⁸ Felix, K., *Z. physiol. Chem.*, 1922, cxx, 94.

⁹ Edlbacher, S., *Z. physiol. Chem.*, 1924, cxxxiv, 129.

IV.

Growth-Promoting Activity.

Dr. Lillian E. Baker kindly tested the growth-promoting activity¹⁰ of the first three of the above samples of hydrolyzed edestin, on sarcomatous fibroblasts. They showed activity which was essentially the same in all three samples. The fourth sample was not tested.

V.

EXPERIMENTAL.

The hydrolyses of edestin were carried out as follows: 3,750 gm. edestin in about 125 cc. water, plus the designated volume of *M* HCl, plus the designated weight (0.2–0.5 gm.) of Armour's 1:10,000 pepsin was allowed to stand the designated lengths of time at 37°C.

After hydrolysis the HCl was neutralized with the same volume of *M* NaOH and the solutions were made up to 150 cc. (2.5 per cent edestin solution). The solutions were heated to 90°C. and filtered from the slight precipitate which formed on heating. These solutions were titrated by adding requisite amounts of dilute acid or base to 5 cc. samples, making up to 10 cc. and determining the pH. The final solutions were 1.25 per cent with respect to edestin, corresponding to a total volume of 300 cc.

¹⁰ Carrel, A., and Baker, L. E., *J. Exp. Med.*, 1926, xlv, 503. Baker, L. E., and Carrel, A., 1928, xlvii, 353, 371.

TABLE II.

Titration Data of Sample EP₁ of Edestin Hydrolyzed with Pepsin.

(6 cc. M HCl; 0.2 gm. pepsin; 3.5 hours at 37°C., giving 5 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	μ	b'	pH	$\frac{b-a}{c}$	μ	b'
2.174	-6.0	0.050	-4.448	6.3	0	0.020	0
2.580	-5.0	0.045	-4.393	6.323	0.25	0.022	0.250
3.161	-4.0	0.040	-3.842	6.663	0.50	0.023	0.500
3.294	-3.5	0.038	-3.384	6.792	0.75	0.024	0.750
3.585	-3.0	0.035	-2.941	7.316	1.0	0.025	1.000
3.819	-2.5	0.033	-2.465	7.653	1.5	0.027	1.500
4.103	-2.0	0.030	-1.982	7.936	1.75	0.029	1.750
4.381	-1.5	0.027	-1.491	8.296	2.0	0.030	2.000
4.755	-1.0	0.025	-0.996	9.061	2.5	0.033	2.497
5.007	-0.75	0.024	-0.748	9.729	3.0	0.035	2.984
5.215	-0.50	0.023	-0.500	10.941	4.0	0.040	3.739
5.472	-0.25	0.022	-0.250	11.561	5.0	0.045	3.905
6.046	-0.125	0.021	-0.125	11.827	6.0	0.050	3.965

TABLE III.

Titration Data of Sample EP₂ of Edestin Hydrolyzed with Pepsin.

(6 cc. M HCl; 0.2 gm. pepsin; 23 hours at 37°C., giving 14 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	μ	b'	pH	$\frac{b-a}{c}$	μ	b'
2.252	-7.0	0.055	-5.698	6.355	0.25	0.022	0.250
2.558	-6.0	0.050	-5.356	6.626	0.50	0.023	0.500
2.792	-5.0	0.045	-4.628	6.989	0.75	0.024	0.750
3.301	-4.0	0.040	-3.885	7.208	1.0	0.025	1.000
3.457	-3.0	0.035	-2.920	7.457	1.5	0.027	1.500
3.892	-2.5	0.033	-2.471	7.905	2.0	0.030	2.000
3.981	-2.0	0.030	-1.976	8.161	2.5	0.033	2.500
4.399	-1.5	0.027	-1.491	8.817	3.0	0.035	2.998
4.771	-1.0	0.025	-0.996	9.805	4.0	0.040	3.982
5.068	-0.50	0.023	-0.492	10.866	5.0	0.045	4.779
5.479	-0.25	0.022	-0.250	11.475	6.0	0.050	5.095
6.16	0	0.020	0	11.814	7.0	0.055	5.015

TABLE IV.

Titration Data of Sample EP₃ of Edestin Hydrolyzed with Pepsin.

(4.5 cc. M HCl; 0.5 gm. pepsin; 23 hours at 37°C., giving 18 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	μ	b'	pH	$\frac{b-a}{c}$	μ	b'
2.724	-6.0	0.052	-5.552	6.636	0.5	0.025	0.500
3.123	-5.0	0.047	-4.826	7.074	1.0	0.027	1.000
3.395	-4.0	0.042	-3.907	7.374	1.5	0.030	1.500
3.685	-3.0	0.037	-2.953	7.634	2.0	0.032	2.000
3.945	-2.5	0.035	-2.474	7.851	2.5	0.035	2.500
4.220	-2.0	0.032	-1.986	8.005	3.0	0.037	3.000
4.443	-1.5	0.030	-1.492	8.174	3.5	0.040	3.499
4.810	-1.0	0.027	-0.996	8.963	4.0	0.042	3.998
5.26	-0.5	0.025	-0.499	9.469	4.5	0.045	4.491
6.2	0	0.022	0	9.946	5.0	0.047	4.973
				10.611	6.0	0.052	5.876

TABLE V.

Titration Data of Sample EP₄ of Edestin Hydrolyzed with Pepsin.

(6 cc. M HCl; 0.5 gm. pepsin; 94 hours at 37°C., giving roughly 30 per cent hydrolysis.)

pH	$\frac{b-a}{c}$	μ	b'	pH	$\frac{b-a}{c}$	μ	b'
3.583	-4.0	0.042	-3.948	6.575	0.50	0.025	0.500
3.869	-3.0	0.037	-2.969	6.946	1.0	0.027	1.000
4.244	-2.0	0.032	-1.987	7.480	2.0	0.032	2.000
4.846	-1.0	0.027	-0.997	8.098	3.0	0.037	3.000
5.213	-0.50	0.025	-0.499	8.544	4.0	0.042	3.999
5.614	-0.25	0.025	-0.250	9.012	5.0	0.047	4.997
5.771	0	0.022	0				

TABLE VI.

Titration of 0.067 Per Cent Pepsin Solution.

(0.2 gm. per 150 cc. mother solution or 300 cc. final solution; treated like the other solutions but containing no edestin.)

pH	$\frac{b-a}{c}$	b'	pH	$\frac{b-a}{c}$	b'
2.491	-1.0	-0.355	7.223	0.08	0.080
2.539	-0.8	-0.222	8.201	0.16	0.160
2.680	-0.6	-0.182	8.580	0.2	0.200
2.927	-0.4	-0.127	10.357	0.4	0.341
3.389	-0.2	-0.118	10.920	0.6	0.386
3.880	-0.1	-0.047	11.167	0.8	0.422
5.408	0	0	11.349	1.0	0.425
			11.631	1.6	0.500

VI.

SUMMARY.

The titration data of edestin show that all the arginine found on hydrolysis exists in this protein as "prearginine."^{1,2}

The extra ionizable groups of histidine, lysine and tyrosine are free in the quantities found on hydrolysis. Part of the extra carboxyl groups of aspartic and glutamic acids are bound as amides, and 50 per cent are bound in some other manner (perhaps anhydride) leaving only about 6 per cent of these groups free to ionize in edestin.

The prearginine in edestin is not converted into arginine on hydrolysis with pepsin up to 18 per cent (of the total hydrolysis). In more highly hydrolyzed solutions it is not possible to detect such a conversion, due to high buffering. Complete hydrolysis however converts prearginine into arginine which can be isolated.

Hydrolyzed edestin promotes the growth of sarcomatous fibroblasts about equally well whether 5, 14 or 18 per cent hydrolyzed.

CHEMICAL ANTAGONISM OF IONS.

I. EFFECT OF Na-Mg AND K-Mg MIXTURES ON THE ACTIVITY OF OXALIC DIION.

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(Accepted for publication, May 20, 1928.)

I.

INTRODUCTION.

It has long been known that various inorganic ions produce characteristic and specific effects on living organisms; and that in so far as the effects of two or more ions are opposite in character, they neutralize (or "antagonize") each other when present in the same solution. In this brief introductory paper it is inadvisable to review the literature on the subject, but it is sufficient to mention that interest particularly concerns four cations, namely; sodium, potassium, calcium, and magnesium.

In general the effect of sodium and potassium is to cause relaxation on one hand and greater permeability on the other. Calcium and magnesium cause contraction and impermeability. But there are also differences between the effects of sodium and potassium and between those of calcium and magnesium.

It has been found that colloids behave differently with different cations. In general calcium and magnesium precipitate sols while sodium and potassium stabilize sols. Antagonism between the effect of univalent and divalent cations on emulsions has been studied by van der Meulen and Rieman¹ and on arsenious sulfide sols by Weiser;²

¹ van der Meulen, P. A., and Rieman, W., *J. Am. Chem. Soc.*, 1924, xlii, 876.
Rieman, W., and van der Meulen, P. A., *J. Am. Chem. Soc.*, 1925, xlvii, 2507.

² Weiser, H. B., Colloid symposium monograph, New York, 1926, iv, 354.

while Höber³ and others have studied antagonism in other colloidal systems. While these are interesting, they deal with unknown factors and have failed to produce a satisfactory explanation of the biological phenomena.

The data below show that a very definite antagonism between sodium and magnesium is found in true (non-colloidal) solutions of oxalate. These agree with simple mathematical formulas. It is hoped they may throw some light on the physiological and on the colloidal antagonisms.

II.

Theory.

In another publication⁴ we showed that the *monoions* of monovalent and polyvalent weak acids in the presence of Na^+ or Mg^{++} ions behave normally in that they obey the limiting Debye-Hückel equation in dilute solution.

Diions and *triions* of weak acids in the presence of Na^+ were found to obey a modified equation in which we introduced a correction for the distance between the like charges. However the presence of Mg^{++} causes marked decrease in the activity of the diion (or triion) which cannot be explained by the Debye-Hückel theory.

TABLE I.

Inactivation of Oxalate Diion by Mg^{++} , as Shown by Conductivity Data.

Data on 0.001 molar MgCl_2 and Na_2Ox solutions and on a mixture of equal parts of these solutions. The ionic strength was equal to $\mu = 0.003$ in all three solutions.

Solution	Conductivity $\times 10^5$	Mean
MgCl_2	21.1	16.5
Na_2Ox	11.9	
1-1 mixture	14.4	14.4
Difference.....		2.1 = 13 per cent decrease

³ Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, Leipsic, 5th edition, 1924, pp. 587-692.

⁴ Simms, H. S., *J. Phys. Chem.*, 1928, xxxii, 1121, 1495.

The deviation of diions with Mg^{++} is small (0.8) for sebacic and azelaic acids. It is greater (1.4 and 8.3 respectively) for succinic and malonic acids, and very great (17) for oxalic acid. The effect on citric acid diion (9) and triion (29) are also large. Oxalic acid was chosen to work with owing to its high deviation and the fact that the difference between its titration indices ($pG_2 - pG_1$) is large enough to greatly simplify the calculations (which are complex enough at best).

Inactivation of dianions with Mg^{++} can be shown by conductivity data, as demonstrated in Table I. The conductivity of a mixture of $MgCl_2$ and $NaSO_4$ solutions is 13 per cent less than the mean of the two solutions.

The antagonism experiments consisted in determining the pH of solutions of oxalic acid containing about 1.5 equivalent of NaOH to which various amounts of $MgCl_2$ and NaCl (or KCl) were added. By calculating the pK' value and comparing it with that of a similar solution with the same ionic strength but containing NaCl alone (*i.e.*, no

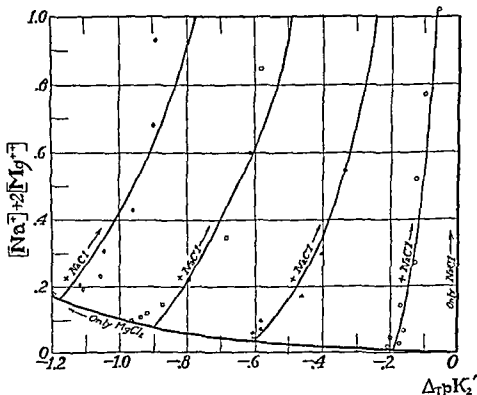


FIG. 1. Effect of $MgCl_2$ and of $MgCl_2$ -NaCl mixtures on pK_2' of oxalic acid (corrected for the "normal" effect produced by the same ionic strength of NaCl). $\Delta TpK_2'$ values are equal to $pK_2' - pK_T'$, see Fig. 2.

MgCl₂), it was possible to determine the effect of Mg⁺⁺ ion on the activity of the oxalate diion.

We found that with a given amount of MgCl₂ the deviation was larger in the absence of NaCl or KCl. Addition of NaCl or KCl caused a decrease in this deviation, and the more NaCl or KCl added the greater the decrease until the value approached the "normal" value (with NaCl or KCl alone). Fig. 1 clearly demonstrates the effect of only MgCl₂ or of MgCl₂ + NaCl.

This constitutes a definite *chemical antagonism between ions* in pure, non-colloidal solution.

III.

Formulation of the Antagonism.

If we assume that the Mg⁺⁺ ion combines with the oxalate diion (Ox⁻) to inactivate it and that the Na⁺ ion (or K⁺ ion) when present interferes with this combination we would expect the following equation to hold:

$$k = \frac{\text{Inactivated Ox}^-}{\text{Active Ox}^-} \times \frac{\gamma_{\text{Na}} + \gamma_{\text{K}} + 2 \gamma_{\text{Mg}}}{\gamma_{\text{Mg}}} \quad (1)$$

The experimental results show that this equation very nearly fits the facts, but that a good constant is given by the equation (where ion concentrations are substituted for activities):

$$k = \frac{\text{Inactivated Ox}^-}{\text{Active Ox}^-} \times \frac{\text{Na}^+ + \text{K}^+ + 2 \text{Mg}^{++} + A}{\gamma_{\text{Mg}} (\text{Ox}^-)^{1.5}} \quad (2)$$

As will be seen in the following section the fraction of Ox⁻ ion inactivated by Mg⁺⁺ is (1 - f') and that not inactivated is f', and we may write the equation as follows:

$$k = \frac{1 - f'}{f'} \times \frac{\text{Na}^+ + \text{K}^+ + 2 \text{Mg}^{++} + A}{\gamma_{\text{Mg}} (\text{Ox}^-)^{1.5}} \quad (3)$$

The introduction of the constant *A* (which changes with oxalate concentration) and of the concentration of oxalate diion raised to the 1.5 power, renders the equation semi-empirical rather than theoretical. The equation is nevertheless useful in proving that the observed an-

tagonism follows definite laws even down to zero concentration of Na^+ and K^+ .

The agreement of the data with equation (3) is summarized in Table II in which the empirical values of A are given and also the calculated values of k . The agreement of the k values may be seen in Tables III, V, VII, and VIII.

TABLE II.

Cation Antagonism.

$$\text{Calculation of } k = \left(\frac{1 - f'}{f'} \right) \frac{\text{Na}^+ + \text{K}^+ + 2 \text{Mg}^{++} + A}{\gamma_{\text{Mg}} (\text{Ox}^-)^{1.5}}$$

Table No.	Cations present	Approximate concentration of oxalate	Empirical constant A	$k \cdot 10^{-4}$
III	Mg^{++} and Na^+	m/100	0.290	15
V	"	m/200	0.060	16
VII	Mg^{++} and K^+	m/100	0.12	9
"	"	m/200	0.02	10
VIII*	Mg^{++} and Na^+	m/100 and m/200	(0.031) and 0.023	22*
" "	Mg^{++} and K^+	"	"	18*

* In Tables III and V the data were obtained from solutions all containing at least 0.0075 mol of Na^+ ion while the solutions in Table VII all contained K^+ ions. In order to show that the equation would hold down to zero concentration of Na^+ or K^+ the data in Table VIII were obtained from a series including solutions free from Na^+ or K^+ and which were found to obey the equation. (The values of k in Table VIII are a little high, but that is unimportant.)

IV.

Physiological Significance.

Data in Table VIII are carried down to zero concentration of Na^+ and K^+ . This point is important since our experimental data are mostly in much higher concentrations of salts than the physiological concentrations. These data show that the same equations hold in the physiological range.

We have demonstrated⁴ two types of deviations of the activity of weak electrolytes caused by the presence of Mg^{++} ions, namely the effect on weak cations and the effect on weak polyanions. We have

also shown⁵ that Mg^{++} ions produce deviations in the activity of proteins (presumably due to both effects). Our present data show that the effect on polyanions is antagonized by Na^+ and K^+ ions and there should be a corresponding antagonism in protein activity.

Such an effect on protein activity may be involved in the physiological antagonisms of ions. Unfortunately we were unable to use Ca^{++} ions in our experiments due to insolubility of calcium oxalate. The effect should be similar to that with Mg^{++} . The following paper will show that a similar antagonism exists between anions (SO_4^- and Cl^-).

We furthermore studied solutions containing $NaCl$ and KCl (but no $MgCl_2$). Although Na and K give slightly different activities, the difference is too small to demonstrate an antagonism in a mixed solution (see Table VI).

V.

Experimental and Mathematical.

A mother solution of oxalic acid was made up containing the indicated equivalents of $NaOH$. To 5 cc. samples of this, varying quantities of 0.25μ and 2.5μ solutions of $NaCl$ (or KCl) and $MgCl_2$ were added and the solutions diluted to 10 cc. (0.01026 molar oxalate). The concentrations of Mg^{++} and Na^+ are given in Tables III to VIII. The concentrations of Na^+ due to $NaOH$ added are included in the indicated values for Na^+ .

b' was calculated from the formula:⁶

$$b' = \frac{b - a}{c} + \frac{h}{c} \quad (5)$$

where h is given by the concentration index (ph) which differs from the activity index (pH) by an amount varying with the ionic strength.⁴

pK_2' is obtained by the equation:

$$pK_2' = pH - \log \frac{\alpha_2}{1 - \alpha_2} \quad (6)$$

where $\alpha_2 = b' - 1$.

⁵ Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 613.

⁶ Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

pK_T' is the index of a similar solution of the same ionic strength and containing NaCl but free from $MgCl_2$. The pK_T' values were observed from a plot of NaCl data given in Table IV. See Fig. 2.

$\Delta_T pK_2' = pK_2' - pK_T'$ is the deviation produced by Mg^{++} ions.

The activity coefficient f' is the antilogarithm of $\Delta_T pK_2'$. It represents the relative amount of active diion. The actual amount

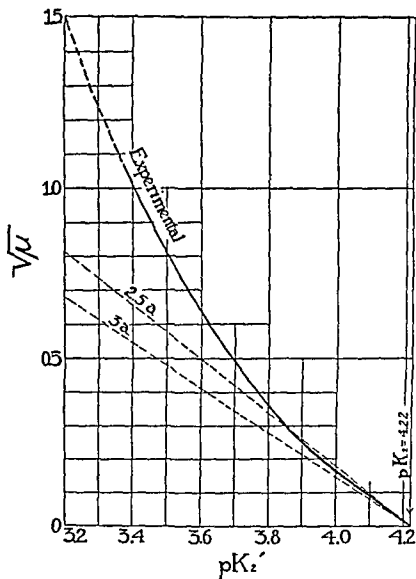


FIG. 2. Effect of NaCl on pK_2' of oxalic acid. This effect is assumed to be "normal" and the abnormalities produced by other salts are measured in terms of their deviation from this curve. Values of pK_2' read from this curve are called " pK_T' ."

We have plotted the values of pK_2' against the square root of the ionic strength ($\sqrt{\mu}$). Curves are also given for the limiting Debye-Hückel equation ($pK_2 - pK_2' = 3a\sqrt{\mu}$) and for the modified equation⁴ ($pK_2 - pK_2' = 2.5a\sqrt{\mu}$).

[illegible]

TABLE IV.
Effect of NaCl Alone on pK_2' of Oxalic Acid.

NaCl added	Na ⁺	$\sqrt{\mu}$	pH	b'	pK_1'
A. (0.0126 molar oxalic acid plus 1.462 equivalents of NaOH plus varying amounts of NaCl)					
0	0.015	0.141	3.989	1.473	4.036
0.013	0.028	0.182	3.930	1.475	3.973
0.025	0.040	0.212	3.901	1.476	3.942
0.050	0.065	0.265	3.841	1.479	3.877
0.125	0.140	0.381	3.754	1.482	3.785
0.250	0.265	0.520	3.653	1.488	3.673
0.500	0.515	0.722	3.543	1.495	3.551
0.750	0.765	0.878	3.460	1.502	3.459
1.000	1.015	1.010	3.409	1.505	3.401
1.125	1.140	1.070	3.385	1.507	3.373
B. (0.00513 molar oxalic acid plus 1.462 equivalents of NaOH)					
0	0.0075	0.100	4.049	1.481	4.082
0.0025	0.0100	0.112	4.035	1.482	4.066
0.0050	0.0125	0.123	4.028	1.483	4.057
0.0075	0.0150	0.132	4.006	1.483	4.035
0.0100	0.0175	0.142	3.989	1.484	4.016
0.025	0.0325	0.187	3.945	1.487	3.967
0.050	0.057	0.245	3.883	1.492	3.896
0.075	0.082	0.292	3.861	1.494	3.870
0.100	0.107	0.332	3.832	1.496	3.835

TABLE V.
Antagonism of Na⁺ and Mg⁺⁺ in Dilute Solutions.

Calculation of k (where $A = 0.060$) in 0.00513 molar oxalic acid plus 1.462 equivalent of NaOH with fixed amounts of MgCl_2 and varying amounts of NaCl.

[illegible]

[illegible]

TABLE VI.

Effect of Mixtures of Na^+ and K^+ in Dilute Solutions.

(0.00513 molar oxalic acid plus 1.462 equivalents of NaOH with fixed amounts of KCl and varying amounts of NaCl).

K^+	Na^+	$\sqrt{\mu}$	pH	δ'	pK_1'	pK_2'	$\Delta_T \text{pK}_1'$
0.0050	0.0075	0.123	4.006	1.483	4.035	4.047	-0.012
"	0.0100	0.132	3.966	1.485	3.991	4.037	-0.046
"	0.0125	0.142	3.978	1.485	4.001	4.022	-0.021
"	0.0150	0.149	3.996	1.484	4.023	4.011	+0.022
"	0.0175	0.158	3.962	1.486	3.986	4.003	-0.017
"	0.0325	0.200	3.934	1.488	3.954	3.949	+0.005
"	0.057	0.255	3.890	1.491	3.905	3.884	+0.021
"	0.082	0.300	3.854	1.494	3.864	3.853	+0.011
"	0.107	0.339	3.817	1.497	3.822	3.819	+0.003
0.0100	0.0075	0.142	3.994	1.484	4.021	4.022	-0.001
"	0.0100	0.149	3.983	1.485	4.008	4.011	-0.003
"	0.0125	0.158	3.974	1.485	3.999	4.003	-0.004
"	0.0150	0.166	3.971	1.486	3.995	3.993	+0.002
"	0.0325	0.212	3.922	1.489	3.941	3.937	-0.004
"	0.057	0.265	3.876	1.492	3.829	3.885	-0.056
"	0.082	0.308	3.851	1.495	3.859	3.847	+0.012
0.025	0.0075	0.187	3.932	1.488	3.952	3.967	-0.015
"	0.0100	0.194	3.925	1.489	3.944	3.958	-0.014
"	0.0125	0.200	3.918	1.489	3.937	3.949	-0.012
"	0.0150	0.206	3.910	1.490	3.927	3.943	-0.016
"	0.0175	0.212	3.915	1.489	3.934	3.935	-0.001
"	0.0325	0.245	3.879	1.492	3.892	3.900	-0.008
"	0.057	0.292	3.864	1.494	3.874	3.858	+0.016
"	0.082	0.332	3.820	1.497	3.825	3.823	+0.002
"	0.107	0.367	3.803	1.499	3.804	3.794	+0.010

8.33	0.14	0.0075	0.187	3.456	1.518*	3.425	3.965	-0.540	0.288	-0.25	0.56	7.37	11
"	"	0.0100	0.194	3.470	1.516*	3.443	3.956	-0.513	0.301	-0.26	0.55	7.39	10
"	"	0.0125	0.200	3.456	1.518*	3.425	3.948	-0.532	0.294	-0.27	0.54	7.42	10
"	0.36	0.0275	0.240	3.475	1.478†	3.513	3.906	-0.393	0.404	-0.31	0.49	6.97	10
"	"	0.040	0.265	3.477	1.478†	3.515	3.885	-0.370	0.427	-0.33	0.47	7.06	9
"	"	0.065	0.308	3.494	1.478†	3.534	3.846	-0.312	0.488	-0.37	0.43	7.30	10
"	"	0.090	0.346	3.467	1.480†	3.501	3.812	-0.311	0.489	-0.41	0.39	7.39	11
"	"	0.140	0.413	3.446	1.482†	3.477	3.760	-0.283	0.521	-0.46	0.35	7.53	9
"	"	0.265	0.543	3.411	1.483†	3.437	3.654	-0.217	0.607	-0.57	0.27	7.86	9
"	"	0.515	0.738	3.357	1.491†	3.372	3.542	-0.170	0.676	-0.68	0.21	8.00	11
Average.....													10
Total average 0.00513 molar* solutions.....													10
Total average 0.01026 molar† solutions.....													9

* 0.00513 molar oxalate, $A = 0.020$.† 0.01026 molar oxalate, $A = 0.12$.

is $f'f \alpha_2 c$ and the actual amount of diion inactivated by Mg^{++} is $(1 - f')f \alpha_2 c$, hence

$$\frac{\text{Inactivated diion}}{\text{Active diion}} = \frac{(1 - f')f \alpha_2 c}{f'f \alpha_2 c} = \frac{1 - f'}{f'} \quad (7)$$

In order to correct the concentration of Mg^{++} ion for the quantity removed in inactivation we must calculate

$$\Delta_0 pK_2 = pK_T' - pK_2$$

where $pK_2 = 4.220$ is the true index in infinite dilution.

The antilogarithm of $\Delta_0 pK_2$ is equal to f or the activity coefficient in the presence of the same ionic strength of NaCl . We then get the activity of Mg^{++} ion from the equation:

$$\gamma_{\text{Mg}} = \text{Mg}^{++} - (1 - f')f \alpha_2 c \quad (8)$$

The value of $(\text{Ox}^-)^{1.5}$ is equal to $(\alpha_2 c)^{1.5}$ in the case of oxalic acid where the titration indices are isolated.

The values of k are calculated by equation (3) (see Tables II to VIII). In this equation we use concentration values of Na^+ , K^+ , and Mg^{++} in the numerator rather than the activity values indicated in equation (1), the latter being unnecessary.

VI.

SUMMARY.

Magnesium ions decrease the activity of divalent organic anions much more than the normal decrease produced by sodium ions. The effect is very large with short chain acids, particularly oxalic acid.

The addition of sodium or potassium ions produces a marked decrease in the effect of magnesium diions on the activity of oxalate diions.

Quantitative data on 0.005 molar solutions of oxalic diion over a wide range of concentrations of MgCl_2 and of NaCl (or KCl) show that the following equation is obeyed:

$$k = \frac{\text{Inactivated Ox}^-}{\text{Active Ox}^-} \times \frac{\text{Na}^+ + \text{K}^+ + 2 \text{Mg}^{++} + A}{\gamma_{\text{Mg}} (\text{Ox}^-)^{1.5}}$$

where A is an empirical value dependent on the concentration of oxalate diion (Ox^{2-}).

This equation has been shown to hold down to zero ionic strength of Na^+ and K^+ , and hence to be valid in the physiological range.

These observations are of biological interest since the activity of proteins should (like oxalic acid) show a similar antagonism.

CHEMICAL ANTAGONISM OF IONS.

II. ANTAGONISM BETWEEN ANIONS AND ALSO BETWEEN CATIONS AND ANIONS IN THEIR EFFECT ON OXALATE ACTIVITY.

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I.

INTRODUCTION.

In the previous paper¹ a chemical antagonism between cations was demonstrated. MgCl_2 produces an anomalous depression in the activity of oxalate diions while NaCl or KCl produces a normal depression. The presence of NaCl or KCl antagonizes the anomalous effect of MgCl_2 according to the semi-empirical equation:

$$k = \frac{1 - f'}{f'} \frac{2\text{Mg}^{++} + \text{Na}^+ + \text{K}^+ + A}{\gamma_{\text{Mg}} \cdot (\text{Ox}^-)^{1.4}} \quad (1)$$

where f' is the fraction of the oxalate diions (Ox^-) inactivated by Mg^{++} and $1-f'$ is the fraction not inactivated.

In this paper we will show that a similar behavior is displayed by Cl^- ions in antagonizing the effect of SO_4^- ions on oxalate ionization (an effect opposite in direction from that of Mg^{++}). We will also show the result of having Mg^{++} and SO_4^- in the same solution.

II.

DISCUSSION.

Previous papers² showed that sulfates cause a rise in the values of pK_1' and pK_2' of malonic acid above the normal indices required by the limiting Debye-Hückel equation.

¹ Simms, H. S., *J. Gen. Physiol.*, 1928, xii, 241.

² Simms, H. S., *J. Phys. Chem.*, 1928, xxxii, 1121, 1495.

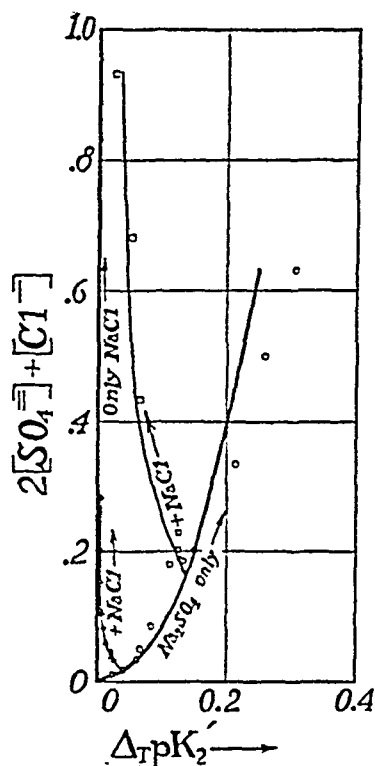


FIG. 1. Effect of Na_2SO_4 and of Na_2SO_4 – NaCl mixtures on pK_2' of oxalic acid.

In the present study we find that the effect of SO_4^{2-} (alone) on pK_2' of oxalic acid increases with the concentration of SO_4^{2-} according to the equation:

$$k = (f' - 1) \frac{2}{\sqrt{\text{SO}_4^{2-}}} \quad (2)$$

This is shown in the right-hand curve of Fig. 1 which is drawn to fit equation (2) and agrees well with the data (circles). The value of k ($=2.6$) is calculated in Table II.

The presence of NaCl antagonizes the effect of Na_2SO_4 as shown in the curves of Fig. 1 for solutions containing fixed amounts of Na_2SO_4 and variable amounts of NaCl . These curves obey the equation:

$$k = (f' - 1) \frac{2\text{SO}_4^{2-} + \text{Cl}^- + A}{(\text{SO}_4^{2-})^{1.5}} \quad (3)$$

TABLE I.
Anion Antagonism.

$$\text{Calculation of } k = (f' - 1) \frac{2\text{SO}_4 + \text{Cl} + A}{(\text{SO}_4)^{1.5}}.$$

Table No.	Anions present*	Approximate oxalate concentration	SO ₄ ⁻ concentration	A	k
II	SO ₄ ⁻ only	m/100	0 to 0.333	0	2.6
III	SO ₄ ⁻ and Cl ⁻	m/100	0.0083	0.015	2.6
			0.0333	0.015	2.6
IV	SO ₄ ⁻ and Cl ⁻	m/200	0.0083	0.36	5.5
			0.0333	2.48	5.2

* Anions present in addition to the oxalate anions.

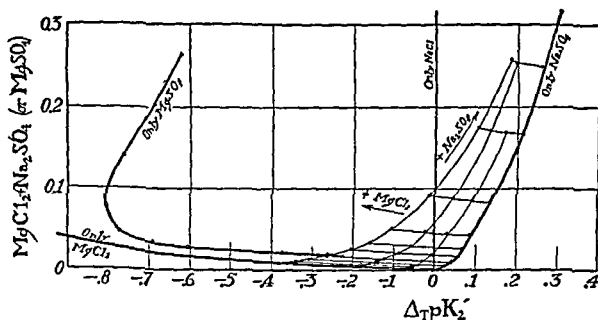


FIG. 2. Effect of Na₂SO₄, of MgCl₂, of MgSO₄, and of Na₂SO₄—MgCl₂ mixtures on pK₂' of oxalic acid.

The MgSO₄ curve is not quantitatively comparable with the other curves due to the difference in amounts of Na⁺ and Cl⁻ present.

The results are summarized in Table I. It will be noted that *A* is practically zero in all m/100 solutions. However in m/200 solution of oxalate *A* varies with the sulfate concentration, and is the only case we have observed in which *A* is not a constant with a

given oxalate concentration. The experimental data are given in Tables II to IV.

The similarity between the first terms of equations (2) or (3) and that of equation (1) is shown by the relation:

$$f' - 1 = \frac{1 - 1/f'}{1/f'} \quad (4)$$

The value of $f' = 10^{\Delta_{\text{rPK}}'}$ is less than unity in the data of equation (1), but greater than unity for the data of equations (2) and (3).

Thus we find that the effect of SO_4^{--} on oxalate activity is antagonized by Cl^- in a manner similar to the antagonism by Na^+ or K^+ of the effect of Mg^{++} .

III.

Solutions Containing Both Mg^{++} and SO_4^{--} .

We have seen that Mg^{++} produces an effect on oxalate activity in one direction and that SO_4^{--} produces a similar (but smaller) effect in the opposite direction. It is interesting to study solutions containing both these anomalous ions. This is shown in Fig. 2 (data in Tables V and VI).

The heavy curves in Fig. 2 show the effect of Na_2SO_4 alone, or of MgCl_2 alone, or of MgSO_4 alone. This last curve is seen to go sharply to the left up to 0.03 molar MgSO_4 , then to turn, and above 0.1 molar point upwards to the right. At first, therefore, the effect of Mg^{++} predominates over that of SO_4^{--} ; but as the concentration of MgSO_4 increases the effect of SO_4^{--} becomes relatively stronger until it predominates and tends to neutralize the initial deviation caused by Mg^{++} .

There is a close similarity between this curve for MgSO_4 and the curves of LaMer² on higher valence types of other ions, an effect which he attributes to the neglect of higher terms in the Debye-Hückel equation.

We furthermore studied the effect of various proportions of MgCl_2 and Na_2SO_4 on oxalate activity. This effect is also shown in Fig. 2 in the lighter curves which form a network. Each curve represents

² LaMer, V. K., and Mason, C. F., *J. Am. Chem. Soc.*, 1927, xlix, 410.

Antagonistic Action of SO_4^{2-} and Cl^- on pK_2' of Oxalic Acid.

Calculation of k (where $A = 0.015$) in 0.01005 molar oxalic acid plus 1.520 equivalents of NaOH with fixed amounts of Na_2SO_4 and varying amounts of NaCl.

[illegible]

TABLE V.

*Effect of $MgSO_4$ Alone on the Activity of Oxalic Diion.*0.01026 molar oxalic acid plus 1.462 equivalents of NaOH, plus varying amounts of $MgSO_4$.

$MgSO_4$	$\sqrt{\mu}$	pH	δ'	pK_1'	pK_2'	$\Delta_T pK_1'$
0	0.142	3.984	1.473	4.028	4.028	0
0.00313	0.180	3.722	1.483	3.751	3.976	-0.225
0.00525	0.235	3.521	1.496	3.528	3.912	-0.384
0.0125	0.265	3.311	1.518	3.280	3.883	-0.603
0.0188	0.308	3.211	1.533	3.154	3.844	-0.690
0.0313	0.381	3.106	1.554	3.014	3.784	-0.770
0.0525	0.520	3.010	1.577	2.875	3.680	-0.805
0.125	0.721	2.952	1.591	2.791	3.552	-0.761
0.188	0.878	2.939	1.592	2.777	3.464	-0.687
0.250	1.010	2.937	1.588	2.782	3.403	-0.621

TABLE VI.

Antagonism of $MgCl_2$ and Na_2SO_4 .(0.01005 molar oxalic acid, plus 1.520 equivalents of NaOH, with varying amounts of $MgCl_2$ and Na_2SO_4 .)

Na_2SO_4	0.00167 M $MgCl_2$				0.00417 M $MgCl_2$				0.00833 M $MgCl_2$			
	pH	δ'	pK_1'	$\Delta_T pK_1'$	pH	δ'	pK_1'	$\Delta_T pK_1'$	pH	δ'	pK_1'	$\Delta_T pK_1'$
0	4.003	1.531	3.950	-0.065	3.896	1.534	3.841	-0.164	3.686	1.543	3.611	-0.381
0.0017	3.994	1.531	3.941	-0.056	3.866	1.535	3.803	-0.179	3.707	1.542	3.634	-0.340
0.0042	3.983	1.532	3.928	-0.040	3.881	1.535	3.820	-0.140	3.714	1.542	3.641	-0.306
0.0083	3.957	1.533	3.900	-0.030	3.866	1.536	3.803	-0.122	3.724	1.542	3.651	-0.263
0.0167	3.937	1.534	3.878	-0.004	3.852	1.537	3.788	-0.089	3.737	1.542	3.664	-0.204
0.0250					3.844	1.537	3.780	-0.058	3.746	1.541	3.675	-0.158
0.0417	3.876	1.536	3.814	+0.030	3.805	1.539	3.757	-0.023	3.744	1.542	3.671	-0.104
0.0833	3.834	1.538	3.768	0.091	3.790	1.539	3.722	+0.046	3.734	1.542	3.661	-0.013
0.167	3.791	1.539	3.723	0.171	3.756	1.541	3.685	0.133	3.724	1.542	3.651	+0.103
0.250					3.735	1.541	3.664	0.200	3.722	1.542	3.649	0.186

V.

SUMMARY.

Sulfate ions (SO_4^{--}) produce an anomalous effect on the ionization of oxalate diion, opposite in direction to the effect of Mg^{++} ions. This effect of sulfate is antagonized by the presence of Cl^- ions according to the equation:

$$k = (f' - 1) \frac{2\text{SO}_4^{--} + \text{Cl}^- + A}{(\text{SO}_4^{--})^{1.5}}$$

where f' is the antilog of the increase in pK_2' due to the sulfate.

In solutions containing up to 0.03 molar MgSO_4 the effect of Mg^{++} predominates over that of SO_4^{--} . Above 0.1 molar the effect of SO_4^{--} predominates and tends to neutralize the initial deviation.

In solutions containing fixed amounts of MgCl_2 and varying amounts of NaSO_4 (or *vice versa*) the effects of these two salts sharply antagonize each other in all proportions.

THE BRIGHTNESS OF THE LIGHT OF THE WEST INDIAN ELATERID BEETLE, PYROPHORUS.

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(Accepted for publication, June 11, 1928.)

The West Indian elaterid beetle, *Pyrophorus*, is one of the brightest of luminous organisms. Pickering, by comparing its light with stars of various magnitudes, believed its intensity to be .004 candle. As Ives has pointed out, it is not so much the intensity, but the specific luminous emission or brightness which is the important quantity in measuring luminescences. The relation between intensity (measured in candles), brightness (measured in lamberts) and specific luminous emission or light flux (measured in lumens) per sq. cm. area of luminescent surface is given by:

$$\frac{\text{candles}}{\text{cm}^2} \times \pi = \frac{\text{lumens}}{\text{cm}^2} \text{ or lamberts.}$$

From Pickering's value, .004 candle, and an assumed light organ area of 1.5 cm.², which is certainly too large, Ives calculated a specific luminous emission of .0084 lumen per sq. cm. or .0084 lambert brightness. By direct measurement, Ives and Jordan obtained .0144 lambert for the glowworm, and Nichols .016 lambert for luminescence of *Cypridina* (an ostracod crustacean) while Dufford, Nightingale and Calvert found .002 lambert for *Cypridina*.

Thanks to Dr. J. S. Dexter of the University of Porto Rico, Rio Piedras, Porto Rico, and to Mr. R. M. Grey, Soledad, Cienfuegos, Cuba, we have recently received some living *Pyrophorus* beetles (Fig. 1), and have measured the brightness of the prothoracic organ by a modified Macbeth illuminometer. Some of the beetles from Porto Rico were 3 cm. long while those from Cuba were 2.5 cm. or less.

Fig. 2 shows one end of a Macbeth illuminometer. A green filter (G) of 24 per cent transmission (measured at the proper color tem-

perature of the illuminometer lamp (I) is placed before the illuminometer lamp (I) and a neutral filter (N) of 9 per cent transmission before the *Pyrophorus* light organ (P). The green filter allows a good color match, while the neutral filter cuts down the light of *Pyrophorus* to a point where readings can be obtained. In addition, a lens (L_1), placed so as to focus the *Pyrophorus* light at the eye (E), serves to fill the comparison field of the illuminometer, a method suggested by

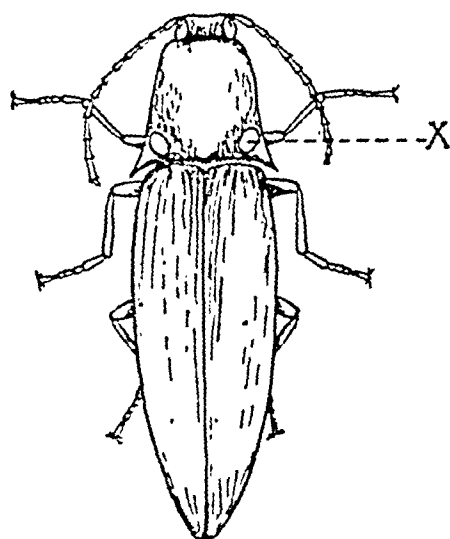


FIG. 1.

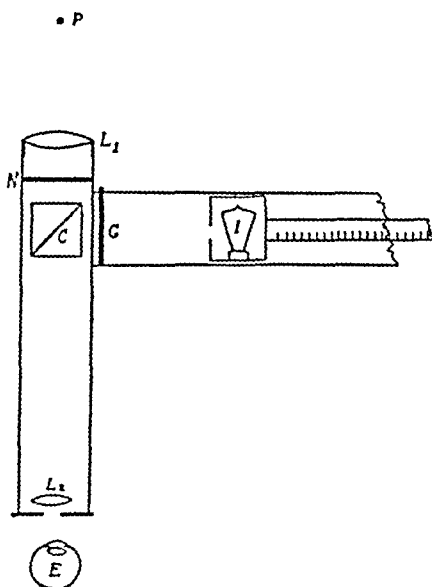


FIG. 2.

FIG. 1. *Pyrophorus* beetle. X , one of the prothoracic light organs.

FIG. 2. P , *Pyrophorus* light organ; N , neutral filter of 9 per cent transmission; G , green glass filter of 24 per cent transmission; C , cube of illuminometer; I , calibrated lamp of illuminometer; L_1 , lens which forms a real image of P at eye E ; L_2 lens for sharp focussing in illuminometer.

Dr. A. H. Pfund, and serving to make determination of equality in brightness of the two fields easy. About half the measurements were made without the lens, but judgment of equality of brightness is not so easy in this case, as the light organ is small.

When the *Pyrophorus* beetles are squeezed between the fingers, their prothoracic organs glow brilliantly for a considerable period of time, long enough to obtain very good readings. There is much varia-

tion in the brightness of various individuals. About 100 observations on 25 beetles gave values on the inverse square scale of the illuminometer of 4 to 22 foot candles, with an average of 8.5 foot candles. Twenty-two observations on 6 beetles which had been fed sugar water and which gave an unusually bright luminescence, averaged 14.3 foot candles with a maximum of 20 foot candles. Taking a high value frequently observed, 20 foot candles, we calculate the lamberts by making allowance for the screens and the reflection factor of the test-plate used to calibrate the Macbeth illuminometer, as follows:

$$20 \text{ foot candles} \times .79 \text{ (reflection factor*)} \times 1.076 \text{ (conversion factor for foot candles to millilamberts)} \times 11.11 \text{ (reciprocal of .09, the transmission of the neutral filter)} \times .24 \text{ (transmission of the green filter)} = 45 \text{ millilamberts.}$$

This value may be taken as nearly correct for an active brightly luminescing beetle. It may be used to calculate the candle power of one organ by the relation given above, if we measure the area of a luminous organ. In the beetle giving the greatest brightness the organ was an irregular ellipse with diameters of .147 cm. and .119 cm. The average diameter is .133 cm. and projected area .0137 cm.² Hence .045 lambert or .045 lumen per sq. cm. $\times .0137 = .00062$ lumen. Divided by π lumens per candle this $= .0002$ candle.

SUMMARY.

The maximum brightness of one of the prothoracic light organs of the West Indian elaterid beetles, *Pyrophorus* (measured at Princeton, New Jersey) was found to be .045 lambert, or .045 lumen per sq. cm. at 20°C. This corresponds to .0002 candle for one organ, or .0004 candle for the pair of prothoracic organs.

* Foot candles is an illumination unit. Multiplied by the conversion factor, 1.076, the illuminated surface would have a brightness expressed in millilamberts, if it reflected 100 per cent of the light. Since no surface does this, the reflection factor (79 per cent) of the particular surface must be used in calculating brightness (millilamberts) when the illuminometer is calibrated in illumination units (foot candles).

ON HEMOCHROMOGEN.

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(Accepted for publication, June 9, 1928.)

I.

The Nature of Hemochromogen.

Introduction.—In a paper (1) concerned mainly with the rôle of the protein, globin, in determining the fitness of hemoglobin as an oxygen carrier, we presented, in a preliminary way, some observations on the nature of hemochromogen. Since then the study of hemochromogen has assumed an entirely new importance. On the one hand, it has been shown by Keilin (10) that substances related to hemochromogen are present in the aerobic tissues of plants and animals generally. On the other hand—as will appear much more clearly in later papers—hemoglobin has proved to be peculiarly suitable material for the study of the denaturation and coagulation of proteins, and when hemoglobin is denatured in alkaline solution in the presence of a reducer, hemochromogen appears. We have accordingly repeated in greater detail and extended our earlier work.

In hemoglobin the simple protein, globin, is combined, in a manner not yet known, with the iron pyrrol complex, $C_{31}H_{32}N_4O_4Fe$ which we call *heme*. The familiar crystalline substance known as hemin is the hydrochloride of heme. The pigment obtained when a reducing agent such as sodium hydrosulfite ($Na_2S_2O_4$) is added to heme we call *reduced heme*. It is necessary to introduce the word heme in order to be able to refer without confusion to a single substance of definite composition.

It was discovered by Stokes (12) that the addition of alkali and a reducing agent to hemoglobin produces a pigment with a well defined two-banded spectrum whose sharpness greatly impressed the noted physicist. Hoppe-Seyler (9) later gave this pigment the name hemo-

chromogen in order to express the *theory*, which has been generally accepted, that the pigment with the two-banded spectrum is identical with the simple iron pyrrol complex, reduced heme.

We define as a *hemochromogen* a substance containing heme whose spectrum has the characteristic pattern described by Stokes. This definition contains no hypothesis about the nature of hemochromogen or about its relation to reduced heme.

It was noticed over 30 years ago by Bertin-Sans and Moitessier (3) that if pure heme is reduced with sodium hydrosulfite, one does not obtain hemochromogen but that hemochromogen is obtained if one adds further to the solution some protein. No precise suggestion was made about the function of the protein. Despite this work the theory of the identity of reduced heme and hemochromogen continued to be accepted. Sodium hydrosulfite was considered to be an "unsuitable" reducing agent. It was well known that hemochromogen could be obtained from pure heme even without any protein by the use of a "suitable" reducing agent such as hydrazine hydrate. Later Dilling¹ (5) and von Zeynek (13) believed pyridine hemochromogen to contain pyridine in addition to reduced heme. Their notion was that pyridine combined with hemochromogen to give a hemochromogen derivative and that pyridine combined with hemochromogen merely because it was a base.

In this paper we propose to show that reduced heme and hemochromogen are different substances with different properties, and that every hemochromogen consists not simply of reduced heme but of reduced heme combined with some nitrogenous substance.

The Preparation and Properties of Reduced Heme and Hemochromogen.

If pure crystalline hemin is dissolved in $\frac{1}{2}$ N NaOH, in which it is readily soluble, and reduced with sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) there results a sparingly soluble pigment, reduced heme, whose spectrum has a diffuse band in the red. When now an excess is added of any of a great variety of substances which *without exception* contain some

¹"Hemochromogen acts as an acid and it becomes evident that it might combine with several bases, page 61."

"Pyridine as a base has the power of combining with the double hematin or hemochromogen radicle to form an ester-like substance, page 60."

nitrogen group, two striking changes occur—the precipitate formed by reducing the heme goes into solution, and the spectrum now shows in place of the diffuse band in the red the two sharp characteristic hemochromogen bands first described by Stokes. One can use for the synthesis of hemochromogen from reduced heme proteins like globin or egg albumin, potassium cyanide, hydrazine hydrate, amines, pyridine, pipiridine, ammonia, pyrrol itself, nicotine, and so on. The hemochromogens described in the past were all obtained in the presence of one or more of these substances.² The “suitable” reducers such as hydrazine hydrate contained nitrogen, the “unsuitable” ones such as sodium hydrosulfite were nitrogen-free. Although all these hemochromogen-forming substances contain some nitrogen group, not all contain an amino group. On the other hand, not all nitrogen-containing substances yield hemochromogen.

Crystallization.—Some, although not all of the hemochromogens can be crystallized. Dilling (5) found that one can readily obtain a permanent preparation of pyridine hemochromogen crystals by dissolving some hemin in a drop of pyridine on a microscope slide, covering the drop with a cover-slip, and then closing it in with Canada balsam and letting the solution reduce “spontaneously.”

We repeated this experiment, using in addition however the reducing agent, sodium hydrosulfite, and obtained the crystals pictured by Dilling.

In the best of such preparations practically all the pigment is in the form of distinct crystals. There is always, however, in addition to the intensely colored crystals a faintly colored background of a very fine precipitate. One cannot see whether this precipitate is crystalline. In order to prove that pyridine hemochromogen can be crystallized it is necessary to show—and this Dilling omitted to do—that the large crystals as well as the fine precipitate give the hemochromogen spectrum, that the crystals are not simply heme whose absorption band is weak.

In the first place, both the crystals and the background are of the same color, a peculiar shade of orange. So they both probably consist of the same substance. We found, furthermore, with the aid of an oil

² Dilling did not realize that the various bases contained in his hemochromogens were all nitrogenous substances, or that nitrogen groups play any rôle in hemochromogen formation.

immersion lens and a Zeiss microspectroscope, that a background free of any visible crystals gave a hemochromogen spectrum much less intense than that given by a single group of crystals with a practically colorless background, one actually much less colored than the control background. This indicates that pyridine hemochromogen whatever its precise nature, is a definite crystallizable compound.

Differences between Hemochromogens.

The hemochromogens as a class, then, have certain characteristic properties in common which distinguish them from reduced heme. They are soluble in alkali and have a sharply defined two-banded spectrum, while reduced heme is only slightly soluble in alkali and has a diffuse spectrum. But although all the hemochromogens are similar in a general way, the precise properties of any given hemochromogen depend on what nitrogen substance is used in its preparation. To designate a particular hemochromogen we therefore prefix to the class name the name of the particular nitrogen substance used in its preparation, thus globin hemochromogen or ammonia hemochromogen.

Spectra.—The most convenient and precise although not the only way to distinguish the various hemochromogens is by means of the exact position of the sharp α band. With the aid of the Hartridge reversion spectroscope (6), one can detect and measure differences so small as two Ångstrom units. For example the α band of globin hemochromogen prepared by the addition of $\frac{1}{2}$ N NaOH and $\text{Na}_2\text{S}_2\text{O}_4$ to hemoglobin has its maximum absorption at about 27 Å. u. more to the red than the α band of ammonia hemochromogen.

Different preparations of hemochromogen from the same nitrogen substance always show the α band in exactly the same position. Particularly interesting here is the fact that the hemochromogen prepared from hemoglobin by the addition of alkali and a reducer is spectroscopically indistinguishable from the globin hemochromogen prepared by the addition to an alkaline solution of heme of a reducer and globin prepared by Schulz's method (11).

Color.—Since the individual hemochromogens differ in the positions and intensities of their absorption bands they must differ in color too. These differences are not in all cases apparent to the unaided eye. In

some cases the color difference is striking; for instance, that between pyridine hemochromogen which is somewhat orange and ammonia hemochromogen which is somewhat purplish.

Reduced heme and hemochromogen under the same conditions have very different properties and are, therefore, different substances. Since some second substance is needed for the preparation of hemochromogen from reduced heme and since all that the varied compounds which can be used as this second substance have in common is the possession of some nitrogen group, we conclude that in hemochromogen reduced heme is combined in some manner with a nitrogen group. This combination would account for the change in properties of the pigment when the nitrogen-containing substance is added. The differences in the nitrogenous substances would account for the secondary differences in the various hemochromogens.

Hemochromogen Equilibria.

We shall now describe a few experiments designed to bring out the nature of the reaction between reduced heme and nitrogen groups.

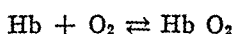
It takes a definite minimum concentration of nitrogenous substance to convert reduced heme into hemochromogen, that is, to produce a soluble pigment whose spectrum shows only the two sharp bands of hemochromogen and none of the characteristic absorption of reduced heme. This minimum concentration varies a great deal from one nitrogen substance to another. In no case is the number of nitrogen groups required less than the number of heme molecules. In some cases it is very much greater. For instance a 3 per cent solution of ammonia is not sufficient to convert reduced heme completely into hemochromogen.

If a solution of ammonia hemochromogen is evacuated, gaseous ammonia is pumped off and the spectrum of reduced heme replaces that of hemochromogen.

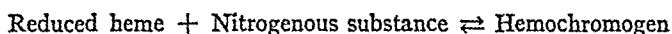
If 5 cc. of 28 per cent ammonia is added to 0.1 cc. of a 3 per cent solution in $\frac{1}{2}$ N NaOH of globin hemochromogen prepared from hemoglobin, the spectrum of globin hemochromogen is replaced by that of ammonia hemochromogen whose α band is about 27 Å. u. further towards the blue.

In three ways, therefore, the reaction between reduced heme and

nitrogen compounds is analagous to the reaction between hemoglobin and oxygen or carbon monoxide. It requires a definite concentration of free oxygen or carbon monoxide to give oxyhemoglobin or carbon monoxide hemoglobin. Likewise a definite concentration of nitrogen compound is needed to give hemochromogen. When an oxyhemoglobin solution is evacuated reduced hemoglobin appears. Likewise when an ammonia hemochromogen solution is evacuated, reduced heme appears. When carbon monoxide is bubbled through an oxyhemoglobin solution the spectrum of oxyhemoglobin is replaced by that of carbon monoxide hemoglobin. Likewise when ammonia is added to globin hemochromogen the α band is shifted to the position characteristic of ammonia hemochromogen. We conclude from these facts that just as there is the equilibrium:



there is also the equilibrium:



and that hemochromogen is always partially dissociated into its two components, reduced heme and a nitrogen substance. In the case of hemoglobin the lower the temperature the more the equilibrium is shifted to the right in favor of oxyhemoglobin. We shall see that the same holds true in the case of the hemochromogen equilibrium.

As has just been described, if enough ammonia is added to globin hemochromogen the spectrum of globin hemochromogen is replaced by that of ammonia hemochromogen. If, however, less ammonia is added then the α band of the resulting solution is intermediate between the α band of globin hemochromogen and that of ammonia hemochromogen. The more ammonia is added, the nearer is the band to that of ammonia hemochromogen. These intermediate spectra can be imitated by means of optical mixtures of the spectra of the two hemochromogens such as are observed by looking at the same time through cells containing separate solutions of the two hemochromogens.

The mere fact that the α band of the solution becomes indistinguishable from that of NH_3 hemochromogen when enough ammonia has been added does not mean that all the globin hemochromogen has

disappeared. A small amount of globin hemochromogen cannot be detected spectroscopically in the presence of sufficient ammonia hemochromogen.

II.

The Reactions between Reduced Heme and Cyanide.

We shall now discuss the reactions of a particular nitrogenous substance, cyanide, with reduced heme. These reactions have a peculiar interest because due to cyanide's great affinity for reduced heme these reactions provide a means of determining the composition of cyan-hemochromogen and because cyanide is able in small concentration to inhibit tissue respiration.

The Two Cyanide Compounds.

Two distinct pigments can be obtained by adding cyanide to reduced heme (1) cyan-hemochromogen which has not hitherto been described and (2) what will be provisionally called the second cyanide compound of reduced heme. This non-committal name is given because the nature of the compound is not yet certain. If the concentration of cyanide is low enough one gets only cyan-hemochromogen, if high enough only the second compound. Intermediate concentrations of cyanide give mixtures of the two pigments. Cyan-hemochromogen has two typical hemochromogen bands designated α and β . The α band is much more intense than the β and has its maximum absorption at about 5535 Å. u. The spectrum of the second compound likewise has two sharp bands, in this case of about equal intensity. The α band is about 150 Å. u. toward the red of the corresponding band of cyan-hemochromogen. In mixtures of the two pigments the two α bands do not fuse. From the relative intensities of these two bands one can, therefore, obtain a rough idea of the relative concentrations of the two components of the mixture.

The Composition of Cyan-Hemochromogen.

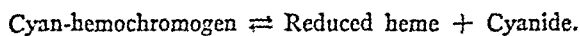
The difficulty of isolating hemochromogen free of uncombined reduced heme and nitrogenous compound led to an attempt to determine the composition of hemochromogen by an indirect method. The following experiments are designed to determine how much cyanide

has to be added to reduced heme to convert it into hemochromogen, that is, to cause the complete replacement of the characteristic spectrum of reduced heme by that of hemochromogen. Four solutions of 2×10^{-4} M hemin in 1 per cent Na_2CO_3^* are made up to contain .65, .75, 1, and 1.25 molecules of KCN per molecule of heme. After addition of the reducer $\text{Na}_2\text{S}_2\text{O}_4$, layers 1.5 cm. thick are examined spectroscopically. Solution .65 shows the α band of cyan-hemochromogen and marked reduced heme absorption in the yellow; solution .75 more hemochromogen and less reduced heme; and solution 1.25 no reduced heme but in addition to cyan-hemochromogen a small amount of the second cyanide compound. Since one molecule of cyanide is adequate to convert one molecule of heme into hemochromogen, cyan-hemochromogen cannot contain more than one cyanide group per heme.

The following control experiment shows that were 25 per cent of the pigment in solution 1 reduced heme, the reduced heme would be detectable spectroscopically, despite the presence of the hemochromogen. Solution 1 contains 2×10^{-4} M reduced heme in 1 per cent Na_2CO_3 plus 2×10^{-4} M KCN. Another solution is made up which contains $.5 \times 10^{-4}$ M or 25 per cent as much reduced heme and no cyanide. The two solutions, each 1.5 cm. thick, are placed together in front of the spectroscope and observed at the same time. One can distinctly see reduced heme in addition to the hemochromogen.

The Affinity of Reduced Heme for Cyanide.

The experiments which have just been described do not exclude the possibility that cyan-hemochromogen contains less than one cyanide group per heme for we have shown that hemochromogen is always partially dissociated into and in equilibrium with its two components, reduced heme and the nitrogenous substance. In the case of the typical hemochromogen, cyan-hemochromogen, there is the equilibrium mixture.



* Na_2CO_3 was used instead of NaOH because, as R. Hill (7) has pointed out, the precipitation of reduced heme is slower in Na_2CO_3 than in NaOH. Our original experiments with NaOH, however, gave essentially the same results.

Thus if a solution of cyan-hemochromogen containing just enough cyanide to cause the disappearance of the reduced heme bands is warmed or diluted, the characteristic absorption of reduced heme reappears. In solution 1, therefore, part of the cyanide is combined with reduced heme as cyan-hemochromogen and part is free serving to drive the equilibrium to the left, to prevent much dissociation of the hemochromogen. If the concentration of free cyanide is small compared with 2×10^{-4} M, that is, if the affinity of reduced heme for cyanide is very great, then the free cyanide in solution 1 can be neglected and cyan-hemochromogen contains neither more nor less than one cyanide per heme. If, however, the free cyanide is not negligible, then cyan-hemochromogen contains less than one cyanide per heme.

Let us assume, although there is as yet no experimental basis for this assumption, that the concentration of free cyanide needed to convert a given fraction of the heme into hemochromogen is independent of the total concentration of the pigment. For any given value of the

fraction $\frac{\text{Reduced heme}}{\text{Cyan-hemochromogen}}$ the lower the total concentration of pigment, the lower the concentration of cyan-hemochromogen or of bound cyanide. The concentration of free cyanide, however, according to our assumption is always the same. Therefore, the lower the total concentration of pigment the greater is the free cyanide relative to the bound. By lowering the pigment concentration enough one can make the free cyanide practically equal to the total cyanide added. This condition is met in the following experiments.

A solution is made up in 1 per cent Na_2CO_3 , 3.75×10^{-6} M in respect to heme and 3.75×10^{-6} M, or 10 times more concentrated, in respect to cyanide. The pigment is reduced with $\text{Na}_2\text{S}_2\text{O}_4$. From the previous experiments the molarity of the bound cyanide is at most equal to the heme with which it is combined, that is at most 3.75×10^{-6} M and, therefore, less than 10 per cent of the total cyanide. The solution is examined spectroscopically through a polarimeter tube 20 cm. long. The intensity of the bands is about the same as that of a more concentrated solution in a narrower vessel. One can see only cyan-hemochromogen—no reduced heme and none of the second cyanide compound. If the cyanide concentration is doubled the

intensity of the α band of the hemochromogen is not visibly increased. If the cyanide concentration is halved, the α band is less intense and one can see reduced heme. To prevent any visible dissociation of cyan-hemochromogen then, when the cyan-hemochromogen concentration is 3.75×10^{-6} N in respect to iron, the concentration of free cyanide must be about 3.75×10^{-5} N.

To discover what differences in cyan-hemochromogen concentration are detectable, three solutions of cyan-hemochromogen are made up in 1 per cent Na_2CO_3 . In all three cases the cyanide concentration is $2 \times 3.75 \times 10^{-5}$ N, that is more than adequate to convert reduced heme into hemochromogen. The heme concentrations are 3.75×10^{-6} N, 25 per cent more, and 25 per cent less. In none of the solutions can either reduced heme or the second compound be seen. The differences in the intensities of the cyan-hemochromogen bands, however, are distinct. Thus in the experiments already described, the formation of 25 per cent additional hemochromogen on doubling the cyanide concentration would be detectable.

The assumption has been made that the cyanide concentration needed to give a definite value of the fraction $\frac{\text{Reduced heme}}{\text{Cyan-hemochromogen}}$ is independent of the total pigment concentration. The percentage of reduced heme which is detectable spectroscopically is likewise roughly independent of the pigment concentration for a decrease in the pigment concentration is compensated for by an increase in the thickness of the layer examined. It follows that the concentration of free cyanide needed to prevent visible dissociation of cyan-hemochromogen is the same, namely about 3.75×10^{-5} , when the total heme concentration (reduced heme plus hemochromogen) is 2×10^{-4} M as in the first experiments as it is found experimentally to be when the total heme concentration is only 3.75×10^{-6} M.

When the total heme concentration is 2×10^{-4} M however, in order to prevent visible dissociation of the hemochromogen, the total cyanide concentration must be 2×10^{-4} M. If the free cyanide is only 3.75×10^{-6} or about $\frac{1}{50}$ of the total cyanide then it is within the rather large experimental error and can be neglected. The bound cyanide can be set equal to the total cyanide.

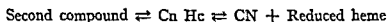
In brief the experiments with the relatively concentrated heme show

that cyan-hemochromogen does not contain more than one cyanide per heme. The experiments with the relatively dilute heme show that reduced heme in alkaline solution has a high affinity for cyanide, and taken together with the other experiments they indicate, although they do not prove conclusively, that cyan-hemochromogen does not contain less than one cyanide per heme.

The Second Cyanide Compound.

The solution containing 1.25 molecules of cyanide per molecule of heme shows in addition to cyan-hemochromogen the second compound. As the cyanide concentration is increased one sees more and more of the second compound and less cyan-hemochromogen. Finally the reduced heme is converted completely into the second compound and the addition of more cyanide causes no further change in the spectrum. Experiments similar to those with cyan-hemochromogen show that the concentration of free cyanide needed to convert reduced heme completely into the second cyanide compound is some 25 times as great as is needed to prevent the dissociation of cyan-hemochromogen.

The factors which determine the composition of the equilibrium mixture:



are the cyanide concentration, the total heme concentration, and the temperature, and the hydrogen ion concentration. The importance of this last factor will be discussed in a later paper. Thus in a mixture of the two cyanide derivatives, more of the second compound appears and less cyan-hemochromogen if first, more cyanide is added; second, the absolute amounts of cyanide and heme being kept constant, the concentrations of both are increased by using less water; and third, the solution is cooled.

The method used to determine the composition of cyan-hemochromogen is likewise applicable to the second compound. Much more free cyanide, however, is needed to prevent its dissociation than the dissociation of cyan-hemochromogen. In order that the free cyanide be negligible therefore in comparison with the bound cyanide, the total pigment concentration must be much greater than is the case with cyan-hemochromogen. In the concentrated solution it is difficult to

determine spectroscopically the precise number of cyanide molecules needed to convert a molecule of reduced heme completely into the second compound. We are prepared to state only that the number is a small one.

Our experiments to determine the relations of the second cyanide compound to other hemochromogens and to carbon monoxide hemochromogen will not be described here because they do not seem capable at present of any simple explanation. This applies especially to experiments in which cyanide is added to reduced heme in the presence of various concentrations of other nitrogen substances.

This investigation of the reactions of cyanide with reduced heme in alkaline solution has shown, so far as we know for the first time, that there are two distinct cyanide derivatives of reduced heme; that one is a typical hemochromogen in which cyanide behaves like a typical nitrogen substance; that reduced heme has a great affinity for cyanide; and that cyan-hemochromogen probably contains one cyanide group per heme.

There have been two attempts to determine the composition of pyridine hemochromogen. The first was made by von Zeynek (13) before the nature of hemochromogen was understood. He found solid pyridine hemochromogen to contain 2.2 molecules of pyridine per molecule of heme. Von Zeynek regarded his single experiment as only preliminary and gave no conclusive evidence that the solid analyzed was pure pyridine hemochromogen. Recently R. Hill (7) has concluded that pyridine hemochromogen contains two pyridine groups per heme. This conclusion is hardly justified by the experiments given and the soundness of the experiments we regard as questionable. Hill's work will be discussed in detail in a separate paper.

III.

*The Reactions between Reduced Heme and Proteins.*³

We shall now consider the reactions between reduced heme and the most complicated of the hemochromogen-forming substances, the proteins. The complication consists in the fact that the individual

³These experiments were described in the doctoral dissertations of the authors which were submitted to Cambridge University in the spring of 1926.

protein molecule contains a large number of free nitrogen groups. One cannot know in advance which and how many of these groups combine with reduced heme. These protein reactions are of special interest because it is by combination with a particular protein, native globin, to form hemoglobin that heme acquires the variety of properties which permit it to act as an oxygen carrier.

Globin.

The first experiments are an attempt to find out how much heme a given amount of globin can convert into hemochromogen. More and more heme is added to the protein until there appears in addition to the hemochromogen bands, the characteristic absorption of reduced heme.

A stock solution of globin hemochromogen is prepared by adding to 1 cc. of 3 per cent horse hemoglobin, 2 cc. of $\frac{1}{5}$ N HCL (which denatures the protein) and then adding NaOH. From this there is prepared a series of solutions all $\frac{1}{10}$ N in respect to NaOH, all containing .033 per cent of globin hemochromogen from hemoglobin, and finally containing in addition varying amounts of extra hemin. On the assumption that the molecular weight of globin is 16,700, the minimum molecular weight, the solutions contain 1, 2.5, 5, 7.5, 10, and 25 molecules of heme per molecule of globin. The solutions are reduced with $\text{Na}_2\text{S}_2\text{O}_4$ and examined spectroscopically.

The differences in the intensities of the hemochromogen bands are striking. The more heme in the solution, the darker and broader the hemochromogen bands. Only in solution 25 can any reduced heme at all be detected. The following experiments show that such results are not given by optical mixtures of reduced heme and hemochromogen such as are observed by looking through two cells at the same time which contain separate solutions of hemochromogen and reduced heme.

Cell 1 contains globin hemochromogen in the same concentration as in the experiments just described, and cells 2 and 3 contain reduced heme, the concentration in respect to heme being 2.5 times greater in cell 2 and 5 times greater in cell 3 than in the hemochromogen solution. Looking through the hemochromogen solution and cell 2

at the same time one can just distinguish reduced heme. When cell 3 is used instead of cell 2 the reduced heme absorption is marked. These experiments show that in the original experiments where reduced heme is added to globin hemochromogen and not kept in a separate cell there did not result a simple mixture of hemochromogen and reduced hemochromogen. One can detect reduced heme in such mixtures.

The fact that the original solution 10 shows no reduced heme and much more hemochromogen than solution 1 therefore indicates that a single molecule of denatured globin can convert at least 10 molecules of heme into hemochromogen.

On adding extra heme to globin hemochromogen two changes can be noted besides the increase in the intensity of the hemochromogen band. Solutions 10 and 25 are hazy and there is a shift of the α band to the red which in solution 25 amounts to about 10 Å. u. The haziness may be due to a decrease in the solubility of the molecule when the globin is loaded with heme; the shift of the band either to a change in the chemical composition of the pigment or in its dispersion.

Since the individual globin molecule seems to have a considerable number of nitrogen groups which can react with reduced heme to form hemochromogen, there must result theoretically, when denatured globin is added to reduced heme, a complicated equilibrium in which a number of nitrogen groups compete for the heme. Practically, however, this complexity is often of no great consequence. The hemochromogen prepared from hemoglobin is of constant composition and is not detectably dissociated into reduced heme at all, any more than acetic acid in strongly acid solution is dissociated—except to a minute theoretical extent—into acetate ions.

Whether extra heme hemoglobin corresponding to the artificial globin hemochromogens which contain extra heme can be prepared is, in the absence of experiments, an open question. Hill and Holden (8) estimate native globin by its oxyhemoglobin-forming capacity. This procedure depends on the tacit and unproven assumption that the composition of the synthetic hemoglobin is independent of the concentration of heme during the synthesis.

Edestin and Zein.

All proteins have not the same hemochromogen-forming capacity. Thus we have found that it takes much more edestin than globin to convert a given amount of heme into hemochromogen and more zein than edestin. These differences are not surprising. We have already seen from a study of the reactions between reduced heme and the simpler nitrogen compounds that the amount of hemochromogen present at equilibrium—the heme concentration and the temperature being kept constant—depends on the number and the nature of the nitrogen groups. It requires a minimum amount either of cyanide or ammonia to convert a given amount of reduced heme into hemochromogen, but it requires much less cyanide than ammonia. In the case of the different proteins the nature of the nitrogen groups and their number per gram of protein varies a great deal. It is not surprising that accompanying these differences in the number and nature of the nitrogen groups, proteins have differences in hemochromogen-forming capacity. The low hemochromogen-forming capacity of zein may be connected with its low diamino acid content (4).

The Difference between Globin Hemochromogen and Hemoglobin.

Based on the classical theory that hemochromogen and reduced heme are identical is the further theory which, so far as we know, has never been questioned that when hemoglobin is converted into hemochromogen the protein, globin, is split off from the iron pyrrol complex, heme. We have shown, however, that globin hemochromogen like hemoglobin itself is a compound of globin and heme. The question thus arises, as to what the difference is between hemoglobin and the hemochromogen obtained from it. We have already (2) pointed out one difference, namely that hemochromogen is a compound of heme and denatured globin while hemoglobin is a compound of native globin. The evidence given for this was that hemoglobin is a typical coagulable protein, and that the denaturation of hemoglobin in alkaline solution as tested for by solubility always runs parallel with formation of hemochromogen as tested for spectroscopically. There may be still other differences between hemoglobin and globin hemochromogen. We do not know whether these two substances have the same molecular weight or whether heme and globin are combined in the same way

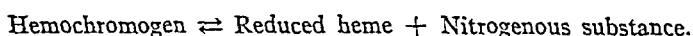
in hemochromogen as in hemoglobin, or in precisely what way they are joined together in either compound.

Hill and Holden (8) have confirmed our view that globin hemochromogen is a denatured protein without seeing fit to mention our paper.

CONCLUSIONS.

1. Every hemochromogen consists of the iron pyrrol complex, reduced heme, combined with some nitrogenous substance.

2. In every hemochromogen there is the equilibrium:



3. Cyanide can form two distinct compounds with reduced heme, one of which is the typical hemochromogen, cyan-hemochromogen.

4. Reduced heme in alkaline solution has a great affinity for cyanide.

5. Cyan-hemochromogen probably contains one cyanide group per heme.

6. The hemochromogen prepared from hemoglobin is a compound of denatured globin and reduced heme.

7. The individual molecule of denatured globin, of hypothetical molecular weight 16,700, can convert at least 10 molecules of reduced heme into hemochromogen.

8. The hemochromogen-forming capacity of globin is, under given conditions, greater than that of edestin, which in turn, is greater than that of zein.

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SYNERESIS AND SWELLING OF GELATIN.

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The name syneresis was given by Graham¹ in 1864 to the phenomenon of the breaking up of jellies on long standing or when disturbed. The jelly then, instead of consisting of one homogeneous mass, becomes segregated into solid lumps surrounded by a thin liquid. Syneresis is quite common in jellies or, as they are called, gels of gelatin, agar, starch, etc. It is possible that the separation of serum from clotted blood and the splitting of soured milk into curd and whey may also be classified under the same term. Various theories have been suggested as to the possible cause of syneresis.

Thus Ostwald² considers syneresis as simply a separation of phases similar to the separation of phases in a critical fluid mixture. Lloyd³ explains syneresis of isoelectric gelatin as being due to the absence of soluble gelatin salts, which otherwise keep the network extended by their osmotic pressure. As it will be seen from further discussion, Miss Lloyd's hypothesis is partly true with respect to the absence of syneresis in gelatin in the presence of electrolytes, although there is apparently some confusion as to the meaning of "soluble gelatin salts." With the exception of some recent work by Liepatoff⁴ on *geranine* and Mukoyama⁵ on *viscose*, very little quantitative work has been done on syneresis in general and of gelatin in particular.

The writer approached the problem of syneresis in gelatin through

¹ Graham, T., Chemical and physical researches (collected papers), Edinburgh 1876, 619.

² Ostwald, W., An introduction to theoretical and applied colloid chemistry, New York, 1917, 93.

³ Lloyd, D. J., *Biochem. J.*, 1920, xiv, 165.

⁴ Liepatoff, S., *Kolloid.-Z.*, 1927, xliii, 396.

⁵ Mukoyama, T., *Kolloid.-Z.*, 1927, xlii, 79.

the study of the swelling of blocks of dilute gelatin when immersed in water. All of the former studies on swelling of gelatin have dealt with the swelling of dry gelatin or of concentrated gels. In all these cases there is always a gain of water and an increase in size of the gelatin block even at low temperatures. Quite different results are obtained when blocks of isoelectric gelatin of concentrations of less than 10 per cent are immersed in cold water. Instead of gaining water the blocks of gelatin lose water, and the lower the concentration of gelatin the greater is the amount of water lost.

Table I shows the values of swelling at 5°C. of gelatin gels of various concentrations when immersed in M/1000 acetate buffer pH 4.7. The

TABLE I.

Swelling of Blocks of Gels of Various Gelatin Content at 5°C. in M/1000 Acetate Buffer pH 4.7.

Electrolyte-free isoelectric gelatin has been used in making up the gels. Gels set for 24 hrs. at 5°C. before they were placed in the buffer solution.

Concentration of gelatin in gm. per 100 cc. solution.....	3.0	4.0	5.0	6.0	8.0	10.0	12.0	14.0	16.0	20.0
Original weight of block of gel (after setting).....	1.610	1.600	1.505	1.790	1.890	1.847	2.020	2.120	2.090	2.250
Final weight of block at equilibrium (after 20-25 days).....	1.150	1.140	1.130	1.435	1.730	1.845	2.200	2.470	2.570	3.050
Per cent change in weight.	-29	-29	-25	-20	-8	0	+9	+17	+23	+36

values are those obtained at equilibrium, which is reached after 20 to 25 days. It is seen that while at concentrations above 10 per cent the gelatin blocks gain in weight, at the lower concentrations there is a continuous drop in weight.

The theories of swelling as developed by Procter, Wilson, Loeb, Northrop and the writer deal with the process of positive swelling of gelatin. These may be summarized as follows:

1. Swelling is mainly a process of osmosis due to a greater concentration of mobile molecules in the block over that of the outside solution in which it is immersed.

2. The molecules causing osmosis may be diffusible ions, as in the case of swelling due to acids, alkalies and some salts, or non-diffusible molecules or groups of molecules as in the swelling of isoelectric gelatin.

3. A block of solid gel behaves like a true elastic body. The swelling is regulated by the elasticity of the gelatin block. At equilibrium the elastic pressure is equal and opposite to the osmotic pressure.

4. When a gelatin sol sets to a gel it is under no elastic strain as long as it is not immersed in water. As soon as it is put into water or salt solution the osmotic forces begin to act against the elasticity of the block causing a strain in the block due to an increase in the bulk. A strain is also brought about in a block of gelatin when water is removed from it by evaporation.

The tendency of a block of a dilute gel to shrink when placed in distilled water indicates that there is still a possible strain in the gel while it sets, which is contrary to the apparent non-strained condition of gels of higher concentration, as demonstrated by Northrop.⁶ With the object of finding a possible explanation of this peculiar behavior of dilute gels the writer undertook a detailed study of the "negative swelling" of gelatin under various conditions.

Experimental Procedure.

All the experiments as well as the weighings were done in a refrigerator room kept at a temperature of about 5°C.

Solutions containing various amounts of isoelectric gelatin in distilled water of pH 4.7 or in salt solutions were heated to 50°C. and coated on weighed microscopic slides (1.5 cc. per slide), or 2 cc. were poured into moulds, consisting of short Pyrex glass tubing of about 15 mm. diameter, which were mounted on a clean paraffin block. The gelatin was allowed to set in the refrigerator for about 20 hours. Care was taken to prevent evaporation by keeping the slides in "moist chambers" or stoppering the tubes with rubber stoppers. After the period assigned for setting, the slides or the blocks, on removal from the tubes, were weighed and put into 150 cc. M/1000 acetate buffer pH 4.7 or into other solutions as described later. After various intervals of time the slides or blocks were dried with filter paper and weighed. In drying blocks of dilute gelatin it was found more convenient to use a clean towel instead of filter paper.

The fluid originally used as the outside solution was distilled water. This was brought to pH 4.7 by means of acetic acid.* But it was soon found that the pH of the water increased slightly after a few days, with the result that the gelatin began

⁶ Northrop, J. H., *J. Gen. Physiol.*, 1926-27, x, 901.

* Distilled water made up with acetic acid to pH 4.7 is designated throughout this paper as H₂O pH 4.7.

to gain in weight instead of reaching a constant value. Hence distilled water was replaced by $M/1000$ acetate buffer pH 4.7.

Fig. 1 shows the effect of two concentrations of acetate buffer pH 4.7 as well as of H_2O on the negative swelling of 4 per cent isoelectric gelatin coated on slides. During the first few days there was a loss of water in all the gelatin blocks; but afterwards those that were kept in

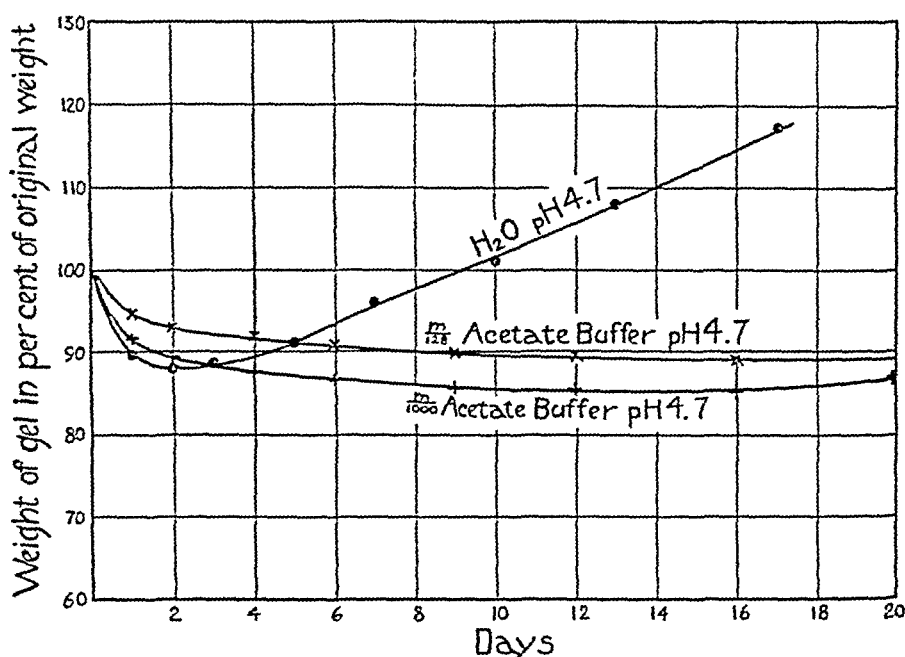


FIG. 1. Effect of a slight change with time in the pH of the outside solution on the change in weight of 4 per cent gels at $5^{\circ}C$. The pH of the distilled water became slightly higher than 4.7 on standing.

H_2O began to gain weight continuously, while the others that were put in buffer solution kept on losing weight until the 10th day when an approximate equilibrium was established. After the 20th day the block in $M/1000$ buffer began to gain slightly in weight but on renewal of the buffer the equilibrium weight was reached again. The experiment also shows that there is less loss in weight of gelatin with the increase in concentration of salt in the surrounding medium. This is simply due to the fact that salts generally increase the swelling of isoelectric gelatin up to a concentration of $M/2$.⁷

⁷ Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925-28, viii, 317.

Is the Negative Swelling Due to Solution of the Gelatin?

A qualitative test on the protein content of the surrounding medium showed that only a trace of the gelatin goes into solution. Quantitative experiments were carried out as follows:

Blocks of 4 per cent of isoelectric gelatin were allowed to remain in large volumes of M/1000 acetate buffer pH 4.7 until the loss in weight became constant. The blocks were then removed, put into weighed evaporating dishes, weighed and then dried in an electric oven at 100°C. for 24 hours. In all cases the dry weight measurements checked with the weight of gelatin as calculated from the loss of

TABLE II.

Effect of Duration of Setting on the Negative Swelling of 5 Per Cent Isoelectric Gelatin in M/1000 Acetate Buffer pH 4.7.

Time of setting	0	16 hrs.	40 hrs.	68 hrs.	92 hrs.	6 days	8 days
Weight of gelatin after being in moist chamber, in per cent of original weight.....	100	99.4	100	101	98.5	95	100
Final weight of gelatin in M/1000 acetate buffer at equilibrium.....	87.0	91.0	90.0	86.0	91.0	88.4	90.0

water. Thus it is clear that while some of the gelatin may go into solution, the amount is too insignificant to affect the results. The negative swelling is due to loss of pure solvent.

Effect of Duration of Setting on the Negative Swelling of Gels.

A number of weighed slides were coated with 5 per cent isoelectric gelatin. The slides were then reweighed and placed over water in rubber-stoppered glass tubes. Care was taken that the gelatin was not in contact with the water. After various intervals of time the slides were removed, weighed and put into M/1000 buffer pH 4.7. The results are given in Table II.

It is seen that the duration of setting has very little effect on the final loss of water from the gel. In addition the experiment shows that the amount of water lost from the gelatin while kept in a closed moist chamber is very small during the first few days.

Effect of Drying of Gels.

A series of slides were coated with 5 per cent isoelectric gelatin solution and then placed for various lengths of time in loosely covered Petri dishes the cover of which had a padding of moist filter paper. This was done in order to prevent too rapid drying of the gels. The slides were afterwards put into M/1000 buffer pH 4.7. Table III shows that the final loss of water is independent of the loss of water due to evaporation. If more water was lost by evaporation than would have been the case had the block been immersed in water, then the gelatin gains water when placed in liquid. In other words, the equilibrium value can be obtained from two directions.

TABLE III.

Effect of Drying on the Equilibrium of Negative Swelling of 5 Per Cent Isoelectric Gelatin in M/1000 Acetate Buffer pH 4.7.

Time of drying, in hrs.	4	16	44	68	92	116
Weight of gelatin after drying, in per cent of original weight.	99.0	97.0	93.0	90.0	87.0	83.0
Final weight of gelatin in M/1000 acetate buffer.	87.0	87.5	87.5	87.5	88.0	87.0

Effect of Volume of Outside Solution or Size of Block on the Equilibrium Value of Negative Swelling.

A 4 per cent solution of isoelectric gelatin was allowed to set for several days in a stoppered flask. No fluid was observed on the surface of the gelatin except for a few drops of condensed vapor on the sides of the flask. A lump of about 10 gm. of gel was then removed by means of a spatula and placed in a weighed porcelain Gooch crucible and its weight determined. The gel was then mashed up by means of the spatula. Fluid immediately began to ooze from the gelatin. Also fluid appeared in the cavity in the stock of gelatin in the flask. The crucible was left to drain overnight on a Gooch funnel. The weight of the gelatin next morning was found to be 82 per cent of the original. The crucible was then placed in a beaker with enough M/1000 acetate buffer to reach the edge of the crucible (about 50 cc.). After several

hours the weight of the gelatin became about 80 per cent and next day it reached the value of 77 per cent, which then became constant. Its dry weight was then determined and was found to check with the weight as calculated from the loss of water. The amount of water lost here by the 4 per cent gel through "syneresis" was identical with the amount of water lost by a solid block of 4 per cent gel weighing 1.5 gm. and immersed in 150 cc. of liquid with several changes of the outside solution. In this last case it took about 10 days to reach the equilibrium state. In another experiment two solid blocks of 4 per cent gel each weighing about 3.5 gm. were placed in stoppered Pyrex tubes, to one of which was added about 5 cc. of H_2O pH 4.7, while the other tube had no water at all. But the pressure of the block of gelatin on the glass was enough to start the diffusion of fluid from the gelatin with the result that the loss in weight of both blocks was identical, each losing 17 per cent in weight after 3 days.

In complete absence of water the process of syneresis is very slow due to the slow rate of diffusion of water through the dry surface of the solid gelatin. A trace of water placed on the surface through actual addition or through pressure is enough to start a rapid diffusion of the water from the solid gelatin. A similar occurrence takes place when a water-permeable collodion bag is filled with water and a moderate pressure applied to it. The rate of diffusion of water from the bag is much greater when the bag is immersed in water than when the bag is placed in a moist chamber after it has been dried carefully with a towel. In an actual experiment the writer found that while it took only about 1 minute for a definite volume of water under a pressure of 10 cm. mercury to diffuse out from a collodion bag when immersed in water, it required about 20 minutes for the same volume of water under the same pressure to diffuse out from the same bag after it was dried outside with a towel and placed in a closed tube *over* water. When the bag was immersed in water again the diffusion became as rapid as before.

The Mechanism of Syneresis.

The experiments thus far described prove that syneresis of gelatin can be conveniently studied by measuring the loss of water in dilute gelatin blocks when kept in dilute buffer pH 4.7 at low temperature,

since the same quantity of water is lost finally whether the block is immersed in a large volume of water or when syneresis is initiated through mechanical forces such as shaking, pressure, etc. Fig. 2 shows the rate of gaining or losing water by isoelectric gels of various gelatin content when kept in M/1000 acetate buffer pH 4.7 at 5°C. The equilibrium values of the concentrations of gelatin in the various gels were plotted separately in Fig. 3 against the original concentra-

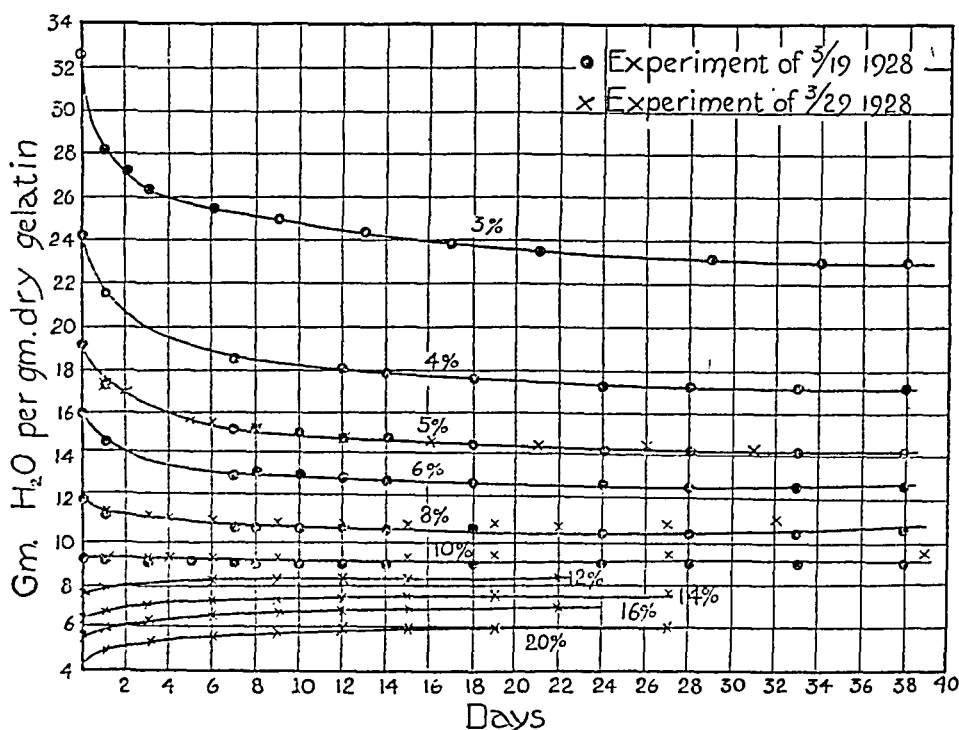


FIG. 2. Swelling of various concentrations of isoelectric gelatin blocks in M/1000 acetate buffer pH 4.7 at 5°C. The gelatin was allowed to set for 24 hours at 5°C. before placing it in the buffer solution.

tions. It is seen that all the points lie on a smooth curve, no matter whether there has been positive or negative swelling, thus suggesting that there is a common mechanism to both forms of swelling. The theoretical relation between the original and final concentrations of a swelling gel has been developed by Northrop⁸ for concentrations of

⁸ Northrop, ⁶ p. 898.

gelatin above 10 per cent. His theory is based on the assumption that a block of gelatin behaves like a perfect elastic body, namely that in accordance with Hooke's law any strain in the block is proportional to the stress producing it. A block of freshly set gel is under no strain

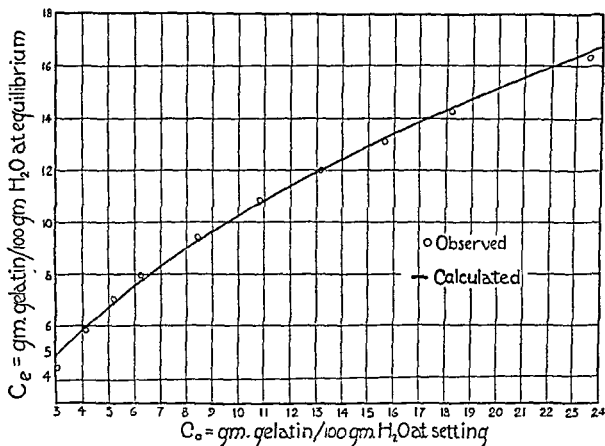


FIG. 3. Relation between the original and the final concentrations of the gelatin in swelling of gels. The dots are the experimental values. The smooth curve represents the theoretical relation:

$$C_e = -\frac{K_s - 140}{26.6} + \sqrt{\left(\frac{K_s - 140}{26.6}\right)^2 + \frac{K_s}{13.3} C_o}$$

where $K_s = 195$.

but as soon as it is placed in water the block swells under the influence of a certain swelling pressure P and a strain is produced in the block. At equilibrium we have

$$P = K_s \frac{V_e - V_s}{V_s} \quad (1)$$

Where K_e is bulk modulus of elasticity of the block and V_o and V_e are cubic centimeters of water per gram of dry gelatin at setting and at equilibrium respectively.

The swelling pressures of gels of higher than 10 per cent have been

TABLE IV.

Application of Northrop's Formulas for Swelling of Gels.

$$\frac{1330}{V_e} - 140 = K_e \frac{V_e - V_o}{V_o} \text{ and } P = \frac{1330}{V_e} - 140$$

K_e = Bulk modulus of elasticity in mm. Hg of pressure.

V_o = cc. H₂O per gm. gelatin at setting.

V_e = cc. H₂O per gm. gelatin at equilibrium.

P = Swelling pressure in mm. Hg.

Concentration at setting of gelatin in gm. per 100 cc. solution	V_o	V_e	P	$K_e = \frac{P \times V_o}{V_e - V_o}$	$C_o = \frac{100}{V_o}$ gm. gel. per 100 cc. H ₂ O	$C_e = -\frac{K_e - 140}{26.6} + \sqrt{\left(\frac{K_e - 140}{26.6}\right)^2 + \frac{K_e C_o}{13.3}}$	C_e observed $= \frac{100}{V}$
3.0	32.5	23.0	-82.2	281	3.07	4.95	4.35
4.0	24.2	17.0	-61.8	208	4.13	5.98	5.88
5.0	19.2	14.2	-47.0	180	5.18	6.90	7.04
6.0	16.0	12.6	-34.5	163	6.25	7.72	7.94
8.0	11.80	10.65	-16.0	164	8.47	9.26	9.40
10.0	9.25	9.20	+4.5		10.80	10.70	10.86
12.0	7.60	8.35	+19.5	198	13.15	11.98	11.98
14.0	6.42	7.60	+35.0	191	15.6	13.20	13.15
16.0	5.50	7.00	+50.0	183	18.2	14.40	14.30
20.0	4.26	6.10	+78.0	181	23.5	16.60	16.40
Average.....				195			

measured directly and found that they can be expressed empirically as the following function of V_e , namely,

$$P = \frac{1330}{V_e} - 140 \quad (2)$$

Combining the two equations we get the following relation between V_o and V_e :

$$\frac{1330}{V_e} - 140 = K_e \frac{V_e - V_o}{V_o} \quad (3)$$

As shown by Northrop, this relation holds well for the swelling of blocks of gels of a gelatin content of more than 10 gm. per 100 cc. H_2O . The same formula was applied by the writer to the cases of negative swelling of gels of lower than 10 per cent with the following results.

Table IV gives the values of K_s as calculated from the known values of V_s and V_o . It is seen that K_s is practically constant, varying as much in the cases of negative swelling as in the cases of positive swelling. Equation (3) can be easily used to find the values of the equilibrium concentrations of the gelatin in the gels in grams per 100 cc. H_2O for the various concentrations used, namely,

$$C_s = -\frac{K_s - 140}{26.6} + \sqrt{\left(\frac{K_s - 140}{26.6}\right)^2 + \frac{K_s}{13.3} C_o}$$

where

$$C_s = \frac{100}{V_s} \text{ and } C_o = \frac{100}{V_o}$$

The calculated values of C_s as well as the observed ones are given in the last two columns of Table IV. They are practically identical with the exception of the lowest concentration. This is also shown clearly on Fig. 3 where the calculated values of C_s are plotted on the smooth curve.

Thus it is evident that the same laws which hold for swelling of gels of a gelatin content higher than 10 gm. per 100 cc. H_2O hold also for the process of giving off water by gels of a lower gelatin content. The active force P in both processes can be calculated by means of equation (1). The values for P are positive in the high concentrations and negative in the lower concentrations, all lying on one straight line which crosses the zero axis at a concentration of gelatin of about 10 per cent (see Fig. 4).

What is Swelling Pressure?

It has been assumed by Northrop and the writer⁹ that the active force P , i.e., the swelling pressure in gels, is brought about by

⁹ Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926-27, x, 162.

osmotic pressure of the soluble ingredient in the gel, and is numerically equal to it. But the fact that the swelling pressure becomes negative in gels of a gelatin content of less than 10 per cent indicates that osmotic pressure is not the only factor which causes swelling, that there is also another force which evidently works in the opposite

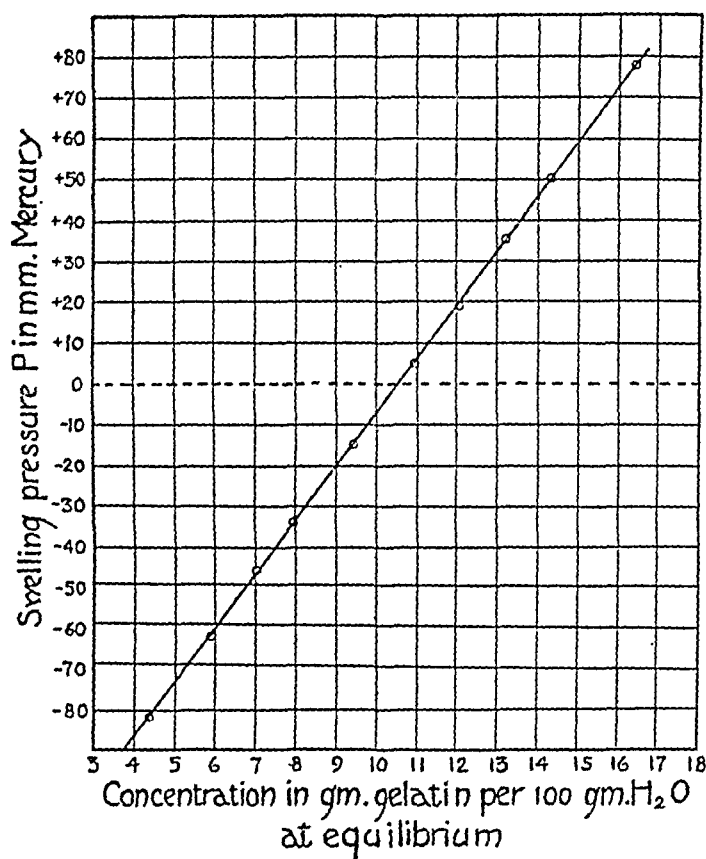


FIG. 4. Swelling pressure of gels of various gelatin content pH 4.7 at 5°C., from the relation $P = 13.3 C_e - 140$.

direction to that of the osmotic force, and that the observed swelling pressure is the resultant of the two oppositely acting forces. It will be shown in the following discussion that the force that causes shrinking of dilute gels is connected with the micellar structure of gelatin solutions and is brought about by the strain prevalent in the micellæ before the gelatin has set to a gel.

Loeb¹⁰ first established the theory that the increase of hydration of gelatin in solution due to a change in pH, as shown by viscosity measurements, is brought about by the swelling of the micellæ in the gelatin solution. The micellæ swell because of a higher osmotic pressure inside of the micellæ than outside due to a difference in the ion distribution in accordance with the law of "Donnan equilibrium." The writer¹¹ has further developed Loeb's idea of hydration by osmosis by applying it to the case of hydration of gelatin at its isoelectric point, where in the absence of diffusible ions no Donnan equilibrium occurs. It was, namely, shown that the hydration of isoelectric gelatin in solution, as demonstrated by viscosity and osmotic pressure measurements, is caused by the fact that each micella in gelatin consists of an insoluble shell containing a definite amount of a soluble ingredient of gelatin. The latter exerts an osmotic pressure on the micella and brings about an inflow of water into it until the osmotic pressure in the micella is balanced by the total osmotic pressure of the solution acting against it and its elastic pressure. The equilibrium state can be expressed as $P_i - P_o = Eq$ where P_i and P_o are the osmotic pressures inside and outside of the micella, respectively, E is a constant proportional to the bulk modulus of elasticity of the micella, and q is the amount of water of hydration per gram of gelatin. At low concentration of gelatin the outside osmotic pressure is small, hence the micellæ take up individually relatively large amounts of water. But as the total concentration increases the opposing outside osmotic pressure increases and the micellæ swell less, with the result that q , *i.e.* the amount of hydration per gram of gelatin gradually becomes less and less. Thus, although the micellæ are at equilibrium with the outside solution, they are still under a strain exerted by a pressure equal to Eq , the magnitude of which decreases with the increase in the total concentration of gelatin.

The Theory of the Rôle of the Micellæ in Swelling of Gelatin.

The hydrated micellæ in a gelatin solution are kinetically free and are able to exert osmotic pressure as any other particles or molecules.

¹⁰ Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 2nd edition, 1924, 270.

¹¹ Kunitz, M., *J. Gen. Physiol.*, 1926-27, x, 811.

representing the sum of the two pressures at various concentrations of gelatin may be obtained by plotting the observed values for osmotic pressure of various concentrations of gelatin solutions at a temperature above the setting point, and also the values for the elastic strain in the micellæ as obtained from measurements of viscosity of gelatin solutions. Fig. 5 shows the curve for osmotic pressure at 25°C.¹³ plotted as positive, and the curve for Eg (strain in the micellæ) at 35°C.¹⁴ as

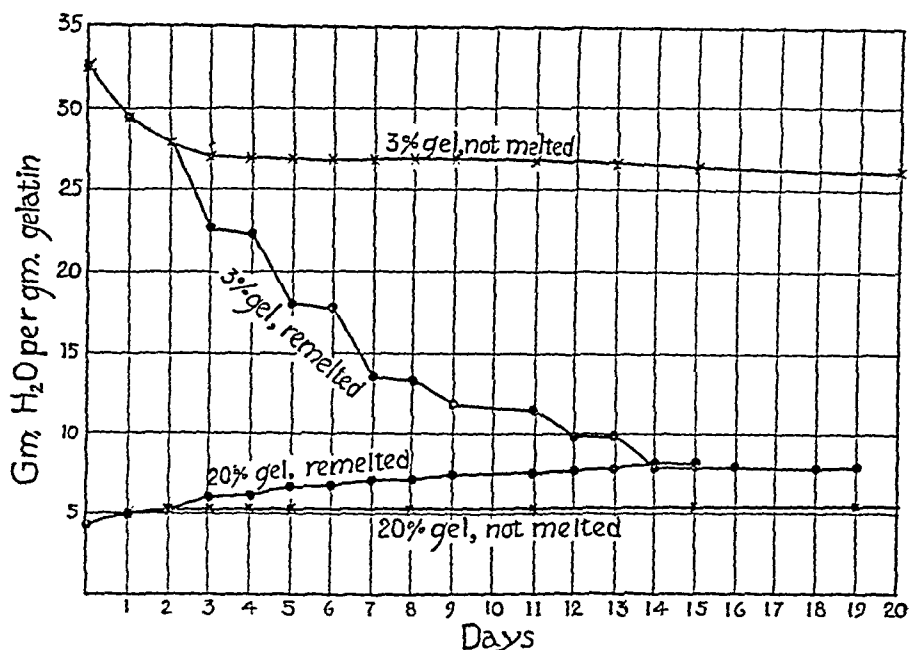


FIG. 6. Effect of reheating of gels on the absorption or liberation of water at 5°C.

negative values of P . The third curve represents the algebraic sum of the two pressures. It resembles closely the actual curve for swelling pressure as shown in Fig. 4.

Effect of Reheating of Gels.

The limiting factor in swelling or in shrinking of gels is the elasticity of the block. When the block of gel is heated the elastic strain caused by the swelling is released. Hence when the gel is cooled again and

¹³ Northrop and Kunitz,⁹ p. 166.

¹⁴ Kunitz,¹¹ p. 820.

put back into water the gel continues swelling until the swelling pressure is balanced by the new elastic pressure. This has been shown by Northrop¹⁵ to hold true for positive swelling. The writer tried the effect of reheating on two gels, one of which was of 3 per cent gelatin content and the other of 20 per cent content. For this purpose the gelatin solutions were coated on slides, allowed to set for 24 hours, weighed and put into $M/1000$ acetate buffer pH 4.7. Every 3rd day the gels were heated carefully over a small alcohol flame until melted. They were then allowed to set for 30 minutes, weighed and put back into the solution. The results are given in Fig. 6. The smooth curves are those of blocks which have not been reheated, while the broken curves are those of the reheated blocks. It is to be noticed that the 3 per cent gel keeps on losing water while the 20 per cent continues gaining water until the concentration reaches about 12 per cent in both cases, where no further change takes place on reheating.

Effect of pH of the Gelatin on the Loss of Water by Gels.

When blocks of isoelectric gelatin are placed in acid or alkali solution they swell enormously due to the setting up of a Donnan equilibrium. This takes place even with very dilute gels. The same thing happens when the gels are made up of gelatin solutions containing acid or alkali. When put into water or acid or alkali the gel swells. It has also been observed by Jordis¹⁶ and noticed by the writer that gels containing electrolytes are quite stable even in dilute solution, and no syneresis occurs. Quite different results are obtained when the acid or alkali is removed by dialysis.

Experiment.—A series of solutions of 3 per cent gelatin were made containing various amounts of HCl or NaOH. The solutions were heated to 50°C., then poured into tubes (2 cc. in each) and allowed to set in a refrigerating room for 24 hours. The blocks were afterwards removed from the tubes, weighed and put into 150 cc. $M/30$ acetate buffer pH 4.7 where they were kept for several days until the weight became constant. The gels were then transferred to $M/1000$ acetate buffer pH 4.7 and kept there until new equilibrium had been

¹⁵ Northrop.⁶

¹⁶ Jordis, E., *Z. Electrochem.*, 1902, viii, 677.

established. The $M/1000$ buffer was renewed several times during the experiment.

The results are given in Fig. 7. It is seen that gels which had a pH other than that of the isoelectric point of gelatin lose more water than gels made of isoelectric gelatin. This shrinking takes place only after the acid or alkali has been removed by neutralization and dialysis. The greater shrinking of gels which contained at setting some acid or

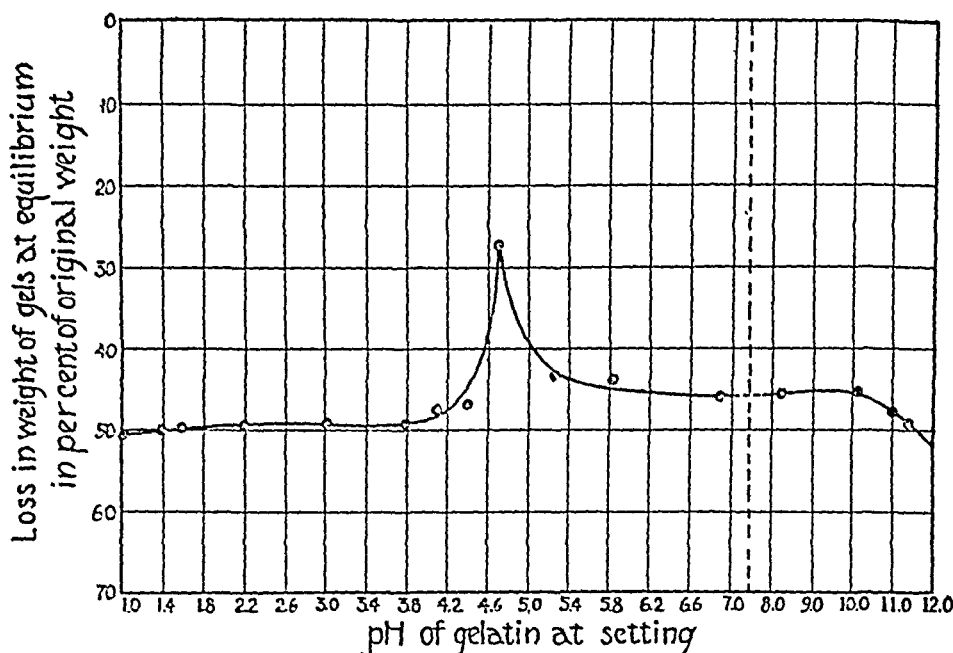


FIG. 7. Effect of pH on syneresis of 3 per cent gel at 5°C.

alkali in solution over those which were electrolyte-free takes place only in gels of low gelatin content. The difference disappears in case of gels of higher concentrations. This is shown in Table V. At a concentration above 6 per cent the loss in water is identical for all gels no matter whether they originally contained acid or alkali or were made up of pure isoelectric gelatin. The peculiar effect of the pH of the original solutions on the loss of water by the gels after the gel was brought back to the isoelectric point becomes clear in view of the theory developed here.

The Theory of Setting of Gels Containing Acid or Alkali.

In dilute solutions of gelatin containing moderate amounts of acid or alkali the micellæ are much more swollen than at the isoelectric point due to a greater concentration of diffusible ions inside than outside of the micellæ. Cooling and setting does not change this unequal distribution of ions. Hence, until the ions are removed by dialysis the micellæ do not lose water and no syneresis takes place. But as soon as the acid or alkali is removed by neutralization and dialysis the micellæ shrink much more than in the case of originally isoelectric

TABLE V.

Swelling of Various Concentrations of Gelatin of Various pH in M/30 Acetate Buffer pH 4.7 at 5°C.

Expressed as per cent of original weight.

Concentration of gelatin in gm. per 100 cc. solution.....			3	4	5	6	8	10
1928								
2/15	pH 9.0.	15 days in M/30 acetate	75	84		99	110	118
2/24	" 4.7.	15 " " " "	95	96		103	108	114
3/10	" 4.7.	2 " " " "	97	98	100	101	106	110
3/10	" 2.0.	2 " " " "	83	90	95	101	106	111
3/10	" 4.7	2 " " " " then 8 days in H ₂ O pH 4.7	79	85	87	88	95	102
3/10	" 2.0	2 days in M/30 acetate then 8 days in H ₂ O pH 4.7	57	70	77	85	93.3	101

gels. With increase in the concentration of gelatin the pH effect on the swelling of the micellæ in a gelatin solution diminishes rapidly, as shown by viscosity measurements,¹⁷ with the result that the pH effect on syneresis in the solid gels is also diminished with increase in concentration of the gelatin. There is also another factor in the effect of acid or alkali on syneresis, namely the solubility effect which is shown also by salts.

The Effect of Salts on Syneresis.

It is generally known that salts affect the solubility of gelatin and the rate of setting (Levites¹⁸). At temperatures above the setting point of

¹⁷ Kunitz,¹¹ p. 832.

¹⁸ Levites, S. J., *Z. Chem. u. Ind. Kolloid.*, 1907, ii, 161.

solutions of gelatin salts split the micellæ into smaller units thus giving rise to higher osmotic pressures (Northrop and Kunitz¹⁹). There is very little action of the salts on the soluble fraction of gelatin, which is in solution even in the absence of salts. The total volume of the micellæ is not changed to any considerable extent, and the viscosity of the gelatin solutions is only slightly increased by addition of salt. As a salt-free dilute solution of isoelectric gelatin is allowed to set the soluble fraction both inside and outside of the micellæ gradually

TABLE VI.

Effect of NaCl on Swelling of 3 Per Cent Gelatin Gels.

Measured after 24 hours in solution.

Weight in per cent of original weight of block.

Concentration of NaCl pH 4.7.....	0 (H ₂ O)	M/256	M/128	M/64	M/32	M/16	M/8	M/4
I. 3 per cent salt-free isoelectric gelatin blocks put into salt solutions	88.6	94.0	95.4		100	102	104	108
II. 3 per cent isoelectric gelatin blocks made up in varying concentrations of NaCl and put into the corresponding salt solutions	86.6	88.5	87.4	89.4	91.2	93.2	94.6	98.4
III. 3 per cent isoelectric gelatin blocks made up in various concentrations of NaCl and put into H ₂ O pH 4.7	90.0	86.0	84.0	82.0	80.0	77.0	73.0	
IV. 3 per cent isoelectric gelatin blocks made up in various concentrations of NaCl. Kept in M/1000 acetate buffer pH 4.7 until equilibrium was established, and then weighed	71.0		60.0	56.0	55.0	54.0		50.0

“precipitates” out during the setting. As stated before, the micellæ lose some of their water of hydration during setting, and the block of gel begins to lose weight almost immediately after it sets if the conditions for diffusion of the water are favorable.

In the presence of salts the setting is slower, first because of the finer state of the micellæ, and secondly because the salts prevent the

¹⁹ Northrop and Kunitz,⁷ p. 332.

precipitation of the "soluble" ingredient of gelatin, as shown by higher swelling of isoelectric gelatin in the presence of salts. The micellæ lose much less water during setting than in the absence of salts. It is possible that the micellæ even take up water during setting in the presence of salts because of the reduction of the outside osmotic pressure due to the immobilization of the micellæ.

When the salt-containing gel is put in H_2O pH 4.7 or dilute buffer of the same pH, the salt dialyzes out and the soluble gelatin both inside and outside precipitates out as in case of originally salt-free gels. The stress on the micellæ due to the greater concentration of soluble

TABLE VII.

Effect of $M/8$ NaCl on Swelling of Concentrated Gels of Isoelectric Gelatin at $5^\circ C$.

Gels were made up in $M/8$ NaCl, allowed to set for 24 hrs. and then put into $M/1000$ acetate buffer pH 4.7. Buffer changed several times.

Concentration of gelatin in gm. per 100 cc. solution.....	10	12	14	16	20
Cc. H_2O per gm. of gelatin at setting.	9.20	7.55	6.37	5.48	4.23
" " " " " " " equilibrium....	9.20	8.30	7.50	6.90	6.00
Per cent increase.....	0	+10.0	+17.6	+26.0	+42.0
Same gels but without salt					
Cc. H_2O per gm. gelatin at setting.	9.25	7.60	6.42	5.50	4.26
" " " " " " " equilibrium....	9.38	8.35	7.60	7.00	6.10
Per cent increase.....	+1.5	+10.0	+18.5	+27.5	+43.0

gelatin inside than outside is removed and the micellæ shrink, thus bringing about a greater loss of water due to syneresis in salt-containing gels than in salt-free gels of pH 4.7, as shown in Table VI. It is interesting to observe that the salt-containing gel, which unlike the salt-free gel is not turbid in appearance, does not assume any turbidity even after the salt is dialyzed out. On the other hand when a milky white salt-free gel is placed in salt solution or acid the gel swells but the turbidity persists, thus proving the turbidity is caused by the structure of the micellæ before setting. The turbidity is apparently caused only by coarse micellæ. The splitting of micellæ into finer elements by salts prevents turbidity formation.

Is the Effect of Salt on Syneresis Due to a Change in the Bulk Modulus of Elasticity of the Block?

A possible explanation of the peculiar effect of dilute salt solutions on syneresis is that the elasticity of the gel is affected by the presence of salt in it and that even after removal of the salt from the gelatin by dialysis the elasticity of the block still remains altered. The change expected would be a decrease in the bulk modulus so that a smaller

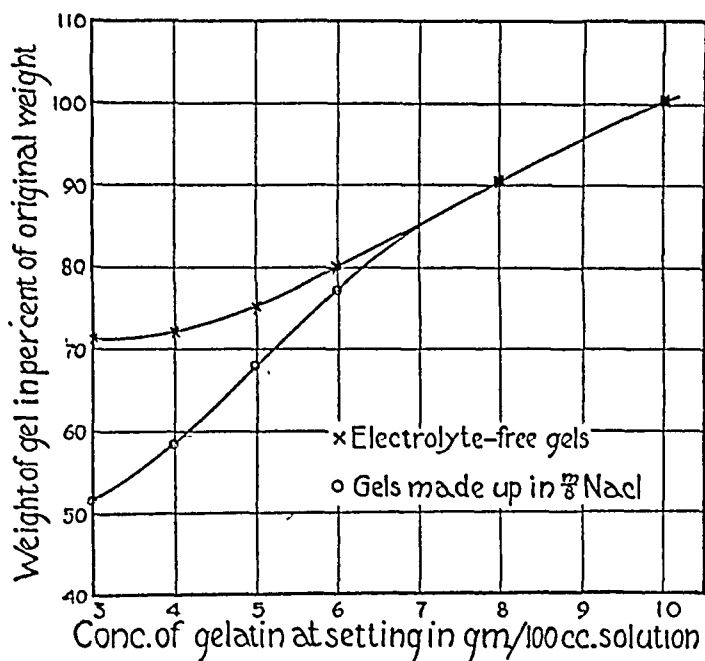


FIG. 8. Effect of salt on syneresis of various concentrations of gelatin blocks in $\pi/1000$ acetate buffer pH 4.7 at 5°C.

force is required to compress the block. But if salts in low concentrations affect the elasticity of a gel then it is to be expected in gels of high gelatin content, that those which originally contained salt and from which the salt was afterwards removed would swell more than gels made up of ordinary isoelectric gelatin. Table VII shows that this is not the case. The swelling of gels of a gelatin content of more than 10 per cent is identical whether the gels originally contained salt or not. The effect of salt is only on the shrinking of gels of low concen-

trations, and it disappears at a concentration of about 8 per cent, as shown in Fig. 8, and is best explainable by the effect of salts on the solubility of the gelatin in the micellæ.

The Solubility Effect of Dilute Acid or Alkali on Syneresis.

Acids and alkalis in addition to their pH effect on gelatin have also an enormous effect on the solubility of gelatin. This explains why the curve for syneresis effect of pH does not give a maximum point similar to the pH viscosity curves. A continuous increase in acid or alkali concentration has the same effect on the solubility of gelatin as addition of large amounts of salt and at higher concentrations of acid or alkali the solubility effect prevails.

SUMMARY.

1. When solid blocks of isoelectric gelatin are placed in cold distilled water or dilute buffer of pH 4.7, only those of a gelatin content of more than 10 per cent swell, while those of a lower gelatin content not only do not swell but actually lose water.

2. The final quantity of water lost by blocks of dilute gelatin is the same whether the block is immersed in a large volume of water or whether syneresis has been initiated in the gel through mechanical forces such as shaking, pressure, etc., even in the absence of any outside liquid, thus showing that syneresis is identical with the process of negative swelling of dilute gels when placed in cold water, and may be used as a convenient term for it.

3. Acid- or alkali-containing gels give rise to greater syneresis than isoelectric gels, after the acid or alkali has been removed by dialysis.

4. Salt-containing gels show greater syneresis than salt-free gels of the same pH, after the salt has been washed away.

5. The acid and alkali and also the salt effect on syneresis of gels disappears at a gelatin concentration above 8 per cent.

6. The striking similarity in the behavior of gels with respect to syneresis and of gelatin solutions with respect to viscosity suggests the probability that both are due to the same mechanism, namely the mechanism of hydration of the micellæ in gelatin by means of osmosis as brought about either by diffusible ions, as in the presence of acid or

alkali, or by the soluble gelatin present in the micellæ. The greater the pressures that caused swelling of the micellæ while the gelatin was in the sol state, the greater is the loss of water from the gels when the pressures are removed.

7. A quantitative study of the loss of water by dilute gels of various gelatin content shows that the same laws which have been found by Northrop to hold for the swelling of gels of high concentrations apply also to the process of losing water by dilute gels, *i.e.* to the process of syneresis. The general behavior is well represented by the equations:

$$P_1 - P_2 = K_e \frac{V_e - V_o}{V_o}$$

and

$$P_1 - P_2 = \frac{1330}{V_e} - 140$$

where P_1 = osmotic pressure of the soluble gelatin in the gel, P_2 = stress on the micellæ in the gelatin solution before setting, K_e = bulk modulus of elasticity, V_o = volume of water per gram of dry gelatin at setting and V_e = volume of water per gram of gelatin at equilibrium.

The writer wishes to express his indebtedness to Dr. John H. Northrop for very valuable suggestions and advice.

THE EFFECT OF THE HYDROGEN ION CONCENTRATION
ON THE RATE OF HYDROLYSIS OF GLYCYL GLY-
CINE, GLYCYL LEUCINE, GLYCYL ALANINE,
GLYCYL ASPARAGINE, GLYCYL ASPARTIC
ACID, AND BIURET BASE BY EREPSIN.

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INTRODUCTION.

The characteristic effect of the hydrogen ion concentration on the activity of erepsin was explained by Euler (1) on the assumption that the enzyme attacked only the ionized form of the substrate. Michaelis (2), on the other hand, has ascribed the effect of the hydrogen ion concentration on the activity of several other enzymes to changes in the ionic species of the enzyme. One of the writers (3) found in the case of pepsin or trypsin that the pH-activity curve varied with different proteins and that the general shape of the curves could be predicted by assuming that pepsin acted upon the positive protein ion and trypsin on the negative protein ion, without making any assumptions in regard to the ionization of the enzyme. The hydrolysis of dipeptides by erepsin furnishes a favorable test of these assumptions since the equilibria governing the substrate are better known than is the case with most enzyme reactions. If the effect of pH depends primarily on changes in the condition of the enzyme, the pH-activity curves for various dipeptides should be the same, while if the pH effect is primarily on the substrate then the pH-activity curves for peptides having different titration curves should be different and should vary with the titration curves of the peptides. The rate of hydrolysis of glycyl glycine (GG), glycyl alanine (GA), glycyl leucine (GL), glycyl asparagine (GApNH₂), glycyl aspartic acid (GAp) and biuret base (B) at various pH values has been determined under

conditions such that the relative rate of hydrolysis (at the different pH values) was independent (or nearly so) of the enzyme concentration and of the substrate concentration. It was found that the pH-activity curve shifts with the titration curve of the substrate so that the condition of the substrate is evidently one of the determining factors. The equilibrium conditions in the substrate, however, are not sufficient in general to enable the entire pH-activity curve to be calculated and it is necessary to assume in addition that the enzyme is a weak acid or base with a dissociation constant of about $10^{-7.6}$ (i.e., $pK' = 7.6$). Independent evidence for this assumption has been found in the effect of the pH on the rate of destruction of the enzyme and the effect of neutral salts on the rate of the reaction. If it is then assumed that the reaction takes place between the ionic forms given in Table I the pH-activity curves may be calculated with a fair degree of accuracy.

TABLE I.

The Ionic Species of Substrate and Enzyme between Which Reaction May Take Place.

Substrate	I. Low pH form of substrate acted on by high pH form of enzyme		II. High pH form of substrate acted on by low pH form of enzyme	
	Peptides	Biuret base	Peptides	Biuret base
Substrate charge	Zwitterion \pm	Cation $+$	Anions — or — — Zwitteranion* $\pm -$	Non-ionized 0
Enzyme charge if an acid if a base	Anion (—) Non-ionized (0)		Non-ionized (0) Cation (+)	

* The dianion (— —) and zwitteranion ($\pm -$) forms refer only to glycyl aspartic acid (of the substances under discussion).

Experimental Methods.

Preparation of Erepsin Solution.—Fresh small intestine of swine was thoroughly washed and run through a chopper several times. The resulting thick paste was mixed with an equal volume of glycerin and toluene was added. It was allowed to stand at 17–20°C. for 3 days and then filtered through cheese-cloth. The resulting solution keeps indefinitely at 0°C. Before use the solution was further

purified by dialysis in a strong, tightly stoppered collodion sac against cold running tap water for 24 hours. A heavy flocculent precipitate forms during this time and is filtered off. The filtrate is again dialyzed for 24 hours against cold tap water and again filtered. This filtrate is used in a final dilution of about 1 to 10. In this dilution the formol titration is negligible and the hydrolysis of 0.02 M GG is half completed in about 2 hours. Dry weight determinations on this solution give a few per cent residue which, however, seems to consist largely of glycerin so that the value is of no significance. The solution has no action on gelatin.

Preparation of the Dipeptides.—The dipeptides, except biuret base, were prepared from racemic amino acids according to Fischer (4) and were recrystallized until the melting points and amino nitrogen figures were correct.

The biuret base was prepared according to Curtius (5). The substance obtained, however, was insoluble in chloroform but in other respects agreed with that described by Curtius. When dissolved in water it gave a solution of about pH 8.0 and when titrated with acid and alkali a titration curve having a pK value of about 8.3 was obtained. On complete hydrolysis with erepsin the equivalent of 3 carboxyl groups were liberated so that it was assumed that the substance was a mixture of the free base and the carbonate. Lack of material prevented more definite identification.

Method of Following the Reaction.—In order to determine the pH-activity curve it is necessary to carry out the reaction at constant pH. The pH may be kept constant by the use of phosphate buffers or by using only the first part of the curve. In the latter case the peptides serve as the buffer if in addition M/100 Na acetate is added to buffer the range from 5.0 to 7.0. Both methods were used at first and it was found with glycyl glycine that the results were the same. The presence of phosphate retards the reaction and as it was later found necessary to work with as dilute erepsin as possible the later experiments were done in the absence of phosphate and the reaction followed only until 20 per cent complete. The pH was measured electrometrically before and after the experiment and was found not to vary sufficiently to affect the results.

Method of Titration. Phosphate Present.—Sufficiently large samples were taken, depending on the concentration of the peptide, to give an initial formol titration of 2 to 5 cc. 0.01 M NaOH. 1 cc. 10 per cent ferric chloride solution was added and sufficient 0.2 M NaOH to make the solution alkaline to phenolphthalein. The phosphate, iron and any protein present were thus precipitated and filtered off. The filtrate was brought to pH 5, 1 cc. formalin added and the solution titrated as previously described (6).

In the absence of phosphate the formalin was added and the sample titrated direct. All titrations were made in triplicate and usually agreed within 0.05 cc. 0.01 M NaOH. Six to ten complete series of hydrolysis at intervals of about 0.5 pH from 5 to 9 were run on each peptide.

Temperature.—All experiments, except those on the rate of inactivation of the enzyme, were made at 25°C.

Method of Calculation, etc.—According to the simple theory of a catalytic reaction the course of the reaction is predicted by the monomolecular formula and the velocity constant obtained in this way should be independent of the substrate concentration and proportional to the catalyst concentration. In the case of enzyme reactions this is not usually the case, the velocity constants decrease as a rule as the substrate concentration increases. If this effect of the substrate concentration on the velocity constant depended on the pH of the solution it is evident that different pH-activity curves would be obtained depending on the concentration of substrate. To be significant the curves must depend only on the pH and be independent of the substrate concentration. It was found in the case of glycy l leucine that the velocity constants decreased much more rapidly with increasing substrate concentration at pH 8.5 than at pH 6.0 so that the relative rate of hydrolysis at pH 8.5 compared to pH 6.0 decreased with increasing glycy l leucine concentration. In order to avoid this difficulty it was necessary to carry

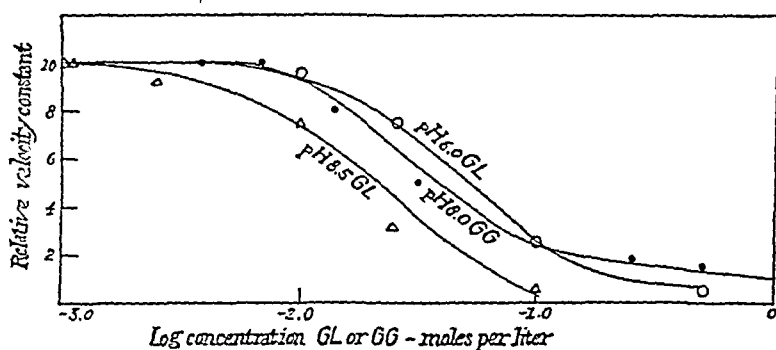


FIG. 1. Effect of concentration of glycy l leucine and glycy l glycine on velocity constant at various pH values.

out the experiments at as low a concentration as possible. The experimental limit for the methods used is about 0.01 M and this concentration was used throughout. In the case of glycy l glycine the effect of concentration was less marked. The results of the experiments on this point are shown in Fig. 1.

Enzyme Concentration.—von Euler and Josephson have noted that the inhibitory effect of glycine on trypsin is marked at pH 8.5 and negligible at pH 6.0 (7). Evidently then, if the enzyme preparation used contains such inhibitory substances, the more concentrated the enzyme solution the more the velocity of hydrolysis in the alkaline range will be slowed down compared to that at pH 6.0. This effect was in fact found to be marked with the glycerin extract if used direct or after partial dialysis but after the double dialysis described was too small to be detected. The presence of such effects can be tested for by diluting the enzyme with an inactivated portion of the same solution. The result of an experiment

with partially purified enzyme solution is shown in Fig. 2. The inactive enzyme markedly inhibits the reaction at pH 7 to 8 and has no effect at pH 6.

Extent of Hydrolysis.—It was found by Levene and Simms (8) that the hydrolysis of sarcosyl glycine, glycyl sarcosine, sarcosyl sarcosine, alanyl glycine, alanyl alanine, etc. did not go to completion but that there was an equilibrium between hydrolysis and anhydride formation. It is therefore necessary to consider the possibility that the position of the end-point depends on the pH. Experiments with excess enzyme, however, show that this is not the case but that the same final amount of hydrolysis takes place at all pH values. Complete hydrolysis occurs with all the peptides studied in this paper except glycyl d-l-leucine in which case the hydrolysis stops at 50 per cent, due either to anhydride formation or to the hydrolysis of only one isomer.

It was also found that fairly good monomolecular constants were obtained, except in the case of glycyl alanine which gave decreasing constants as the reaction

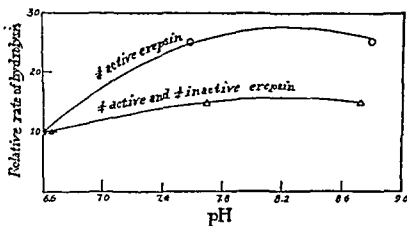


FIG. 2. Effect of concentration of erepsin on pH-activity curve. $\pi/50$ biuret base.

progressed. This is probably due to the fact noted by von Euler (7) that alanine has a more marked inhibitory effect than the other amino acids. The fact that the constants did not decrease with time show that under the conditions used, inactivation of the enzyme is negligible.

As a result of these experiments it may be said that the monomolecular constants for the hydrolysis, when run with purified enzyme solution and with dilute substrate, are a measure of the effect of the pH on the reaction and are independent of the substrate concentration below the value used and of the enzyme concentration provided this is the same in the experiments compared.

In running the experiments a series of peptide solutions were made up and titrated with NaOH so as to differ by about 0.5 pH from each other over the range from pH 4.5 to 10.0. They were then allowed to come to the desired temperature in the water bath and the enzyme solution added. Samples were then taken at 0.5, 1, 2, 4 hours etc. until the reaction was about 25 per cent complete. The

velocity constant was then calculated from the increase in formol titration by means of the formula

$$C = \frac{1}{t} \log \frac{A}{A - X} \quad (1)$$

where A is the maximum increase noted with excess enzyme. The largest velocity constant so obtained was taken as 100 and the other constants expressed in terms of this. Six to twelve experiments were run with each peptide and the final figures obtained by averaging the separate values found at the same pH in different experiments.

The results of the experiments are shown graphically in Fig. 3. The experimental points are represented by circles the size of which shows approximately the magnitude of the probable error of the mean. The solid dots are from von Euler and Josephson (7) and agree well with the present figures. The curves were calculated by means of the assumption described below.

It is evident from the shape and position of the curves that the known equilibria in the substrate solutions are insufficient to account for the results. On the other hand, the fact that the optimum varies with the different peptides shows that the reaction depends to some extent on the condition of the substrate. If, however, the enzyme is assumed to be a weak acid or base with dissociation index (pK') of about 7.6 the curves may be predicted.

There are two possibilities either or both of which may lead to the pH optima found with the various substrates. Referring to the ionic species of the enzyme predominating above or below its dissociation index ($\text{pK}' = 7.6$); or the ionic species of the substrates predominating above or below the indices given in Table II, it may be said that (I) the form of substrate predominating at low pH is acted on by the form of enzyme predominating at high pH, or that (II) the form of substrate predominating at high pH is acted on by the form of enzyme predominating at low pH, or that (III) both these reactions take place.

Other reactions must take place to a much smaller extent to agree with the observations.

The physical significance is clearer if the ionic species involved are considered, as summarized in Table I. In Table II it will be seen

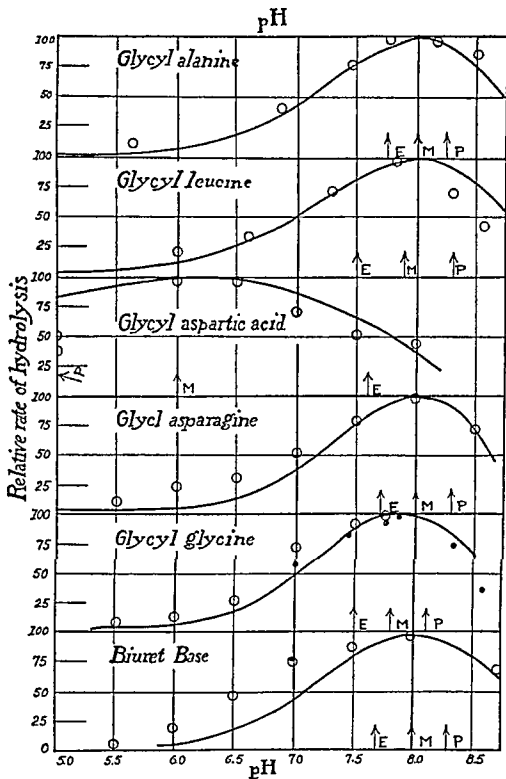


FIG. 3. Effect of pH on the rate of hydrolysis of various peptides.

○ observed.

— calculated.

The arrows indicate: pK_s of substrate, P; the observed maximum, M; and the corresponding value of pK_s (equation 7). Average pK_s = 7.6.

that the four simple ampholytes change from "zwitterion" at lower pH to anion at higher pH (than the pK_s involved in the optimum).

Glycyl aspartic acid, on the other hand, changes from zwitterion to zwitteranion at pH 4.45 which is the pK_s involved in the optimum. However its change from zwitteranion to dianion at pH 8.60 is not accompanied by a noticeable change in reactivity.

Biuret base, on the other hand, changes from cation to non-ionized molecule.

There is no evidence which permits a decision between assumption I and assumption II. According to I the reactive substrate forms have in common one ionized amino group—but so has the zwitteranion of glycyl aspartic acid which, according to this assumption, is not acted on (perhaps due to its negative charge if the enzyme is an acid).

TABLE II.

Calculation of pK_s and Predominating Ionic Species at Low and High pH.

	Form pre- dominating below pK_s	Form pre- dominating above pK_s	pK_s	H_{max}	pK_e calculated
GA.....	±	—	8.25	8.0	7.7
GL.....	±	—	8.29	7.9	7.5
GAp	±	±—	4.45	(6.0)	(7.6)
GApNH ₂	±	—	8.3	8.0	7.7
GG.....	±	—	8.07	7.8	7.5
Biuret base.....	+	0	8.3	8.0	7.7
Mean.....					7.6

According to assumption II the anions of the substrate would be acted on. But biuret base is an exception, it being non-ionized at high pH. If then a non-ionized amino group is necessary for reactivity glycyl aspartic acid is an exception.

It is equally uncertain whether the enzyme is a weak acid or a weak base. Neutral salts have an exponential negative effect on the reactions which according to Brönsted indicates reaction between two oppositely charged ions. However, the activity of weak bases and ampholytes has been shown to be anomalous (14) and the significance of the effect of neutral salt is doubtful.

The formulation of the reaction is the same on the basis of any of the above assumptions, the only difference being the significance attached to the various constants. It will be assumed for purposes of calculation that the enzyme is a weak base and that the reaction is between the negative monovalent ion of the peptides or the non-ionized form of the biuret base and the non-ionized form of the enzyme. Let

$$(HA) = \text{undissociated substrate}$$

$$(HE) = \quad \quad \quad \text{enzyme}$$

$$B = \text{total substrate} = A^- + (HA)$$

$$D = \quad \text{enzyme} = E^- + (HE)$$

Then

$$\begin{aligned} (HA) &\rightleftharpoons H^+ + A^- & (HE) &\rightleftharpoons H^+ + E^- \\ \frac{H^+ \times A^-}{(HA)} &= K_s & \frac{H^+ \times E^-}{(HE)} &= K_s \end{aligned} \quad (2)$$

and if the rate of reaction is assumed proportional to the product of the negative peptide ion and the non-ionized enzyme, then

$$-\frac{dB}{dt} = CA^- (HE) = \frac{C \cdot D \cdot K_s H^+ B}{(K_s + H^+) (K_s + H^+)} \quad (3)$$

and at constant substrate concentration and pH

$$\frac{C \cdot D \cdot K_s H^+}{(K_s + H^+) (K_s + H^+)} = \frac{1}{t} \log \frac{B_0}{B} = \text{velocity constant} \quad (4)$$

The observed velocity constant will therefore be expressed by the equation

$$C' = \frac{CK_s H^+}{(K_s + H^+) (K_s + H^+)} \quad (5)$$

and the position of the maximum velocity can be obtained by differentiating this expression with respect to C' and H . Setting dC/dH equal to 0 and solving for H_{max} , the following expression is obtained

$$H_{max} = \sqrt{K_s K_s} \quad (6)$$

or

$$\text{pH}_{max} = \frac{\text{p}K_s + \text{p}K_s}{2} \quad (7)$$

Since the values of the dissociation indices pK_e of the substrates are known it is now possible to solve for the value of the dissociation index of the enzyme pK_e . The results of this calculation are shown in Table II. The calculation gives a fairly constant value for pK_e of about 7.6 (*i.e.*, $K_e = 10^{-7.6}$). In order to obtain the entire curve this value of K_e may now be substituted in (5) and the equation solved for the value of the velocity constant C' . In order to compare these figures with those found experimentally it is necessary to express them as per cent of the maximum value. The maximum value found for C' is therefore taken as 100 and the other values calculated in proportion. These figures at the different pH were then used to plot the solid line shown in Fig. 3.

The calculation made above shows that the assumptions used are sufficient to account for the general shapes and position of the curve but involve several constants and are not very convincing in the absence of independent evidence. If the enzyme really has a dissociation index of about 7.6 it should be possible to show that some other property changes at that pH. It was found by Goulding, Borsook and Wasteneys (9) that the effect of the pH on the inactivation of pepsin agreed with the assumption that the enzyme was a weak electrolyte with a dissociation constant near pH 6. It seemed possible that a similar effect could be noted in the case of erepsin. The rate of inactivation of erepsin was therefore determined at various pH. The experiment was carried out in two ways, by determining the amount of enzyme in the solution after varying lengths of time by means of the rate of hydrolysis of glycyl glycine and also by following the decrease in the rate of hydrolysis of glycyl glycine with time at 35°C.

First Method.—A series of tubes containing the purified erepsin solution and 0.01 M glycine were adjusted to pH values about 0.5 pH units apart over the range of from pH 5 to 10 and put at 25°C. 1 cc. samples were removed at daily intervals and added to a stock solution of 0.1 M glycyl glycine pH 7.8. The rate of hydrolysis of the glycyl glycine and the velocity constant were then determined as usual and the concentration of enzyme assumed to be proportional to the velocity constant. The values obtained in this way for the concentration of enzyme were then plotted against time and the rate of inactivation of the enzyme calculated. The process was found to be approximately monomolecular. The velocity constants of these curves were then assumed proportional to the rate of inactivation.

tion of the enzyme. Inactivation proceeded slowly up to pH 6.5. It then became more rapid becoming constant above pH 9. The sample at pH 9 was half inactivated in about 3 days. This value was then taken as 100 and the other values expressed in proportion.

Second Method.—The hydrolysis of glycyl glycine under conditions such that the enzyme concentration remains unchanged is known to be monomolecular and the velocity constants are proportional to the enzyme concentration. If the experiment is arranged so that the enzyme is being inactivated the velocity constants when calculated for succeeding time intervals will drop and the values for each time interval may be assumed proportional to the average concentration of enzyme over that interval. This procedure furnishes a method therefore for determining the destruction of the enzyme in the presence of the substrate. In order to obtain significant results, however, it is necessary to arrange the experi-

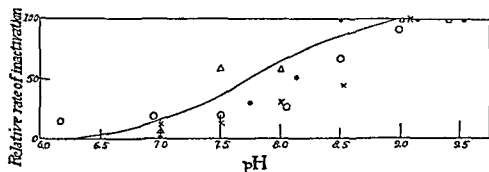


FIG. 4. Inactivation of erepsin at various pH.

— curve calculated from $pK_s = 7.7$.

○ inactivation at $35^\circ + M/100$ glycine.

△ " " $25^\circ + M/50$ glycyl glycine experiment (1).

● " " " " " " (2).

× " " " " " " (3).

ment in such a way that the enzyme is inactivated before the substrate is hydrolyzed to a great extent (10). These conditions are fulfilled by carrying on the reaction with dilute enzyme and concentrated substrate and at a rather high temperature. A series of solutions $0.1 M$ glycyl glycine were therefore adjusted about 0.5 pH apart over the range of from 5 to 10 and placed at $35^\circ C$. The erepsin was added and the hydrolysis followed. A smooth curve was drawn through the figures obtained in this way and the monomolecular constants calculated for short time intervals. These were assumed to be proportional to the concentration of enzyme present at the corresponding time interval. The rate of inactivation of the enzyme could therefore be determined from them as described above.

The effect of the pH on the inactivation of erepsin as determined by these methods is shown graphically in Fig. 4. The solid line rep-

resents the calculated results assuming that the form of the enzyme present in acid solution is very stable compared to the form that is present in alkaline solution and that the value of the dissociation constant is $10^{-7.6}$.

As before it does not matter whether the enzyme is considered as a weak base or acid. The experimental points are irregular but lie in the region of the curve and a little to the alkaline side. The agreement is as good as can be expected for this type of experiment and furnishes independent evidence for the assumption made before that the enzyme had a dissociation curve near pH 7.6.

Calculation of the Rate of Inactivation.

Assume that the enzyme is a weak acid and that the negative ion is very unstable compared to the non-ionized form then

$$\begin{aligned} H^+ + E^- &\rightleftharpoons (HE) & D &= \text{total enzyme} \\ H^+ \times E^- &= K_e(HE) & D &= E^- + (HE) \\ E^- &= \frac{K_e(HE)}{(H^+)} & HE &= (D - E^-) \end{aligned} \quad (8)$$

and

$$E^- = \frac{K_e D}{K_e + H^+} \quad (9)$$

If the rate of inactivation is proportional to the amount of the ionized form then at any constant pH

$$-\frac{dD}{dt} = C' E^- = \frac{C' K_e D}{K_e + H^+} \quad (10)$$

or

$$\frac{C' K_e}{(K_e + H^+)} = \frac{1}{t} \log \frac{D_0}{D} = \text{velocity constant}$$

The reaction will therefore be monomolecular at any pH and the observed velocity constant will be given by the formula

$$C'' = \frac{C' K_e}{(K_e + H^+)} \quad (11)$$

Substituting the value $10^{-7.6}$ for K_a and solving for the value of C'' at different values of H , a series of values are obtained which should be proportional to the observed inactivation constants. In order to compare them the maximum constant value found as the value of H becomes less, is taken as 100 and the values at the other pH expressed in proportion. The solid line in Fig. 4 has been plotted from these figures.

Effect of Neutral Salts.

It was stated above that the results agreed equally well with several assumptions in regard to the ionic species taking part in the reaction. According to Brönsted's (11) theory of chemical reaction velocities the effect of neutral salts on the rate of a reaction differs depending on whether the reaction is between ions of different or like sign or between an ion and a molecule.

The effect of neutral salts on the reaction is complicated by the fact that the presence of salts causes precipitation unless special precautions are taken. This probably accounts for the contradictory literature on the subject (12). The dialyzed glycerin extract becomes cloudy below pH 5 and this precipitation is prevented by the addition of neutral salts. Under these conditions neutral salts slightly increase the rate of hydrolysis. CaCl_2 on the other hand, causes a slight precipitate on the alkaline side of pH 7 and in this range retards the hydrolysis. These effects are presumably secondary and appear to be connected with the formation of the precipitate. It was found by Mr. Johnston that if the erepsin solution was dialyzed in the presence of 0.10 M CaCl_2 and then filtered a solution was obtained which remained clear over the range covered by the experiments. It was less active than the usual preparation. The effect of 0.10 M CaCl_2 on the hydrolysis of 0.01 M GG at various pH using this erepsin solution dialyzed in the presence of CaCl_2 is shown in Fig. 5. The salt inhibits at all reactions but the effect is much more marked on the alkaline side of pH 7 so that the presence of the salt causes a marked shift in the optimum. This result had been obtained by Abderhalden and Fodor (13).

The effect of increasing concentrations of NaCl and CaCl_2 on the rate of hydrolysis of glycyl glycine at pH 7.0 was then determined

with the erepsin prepared as described above. The result is given in Fig. 6 in which the relative rate of hydrolysis has been plotted against the log of the salt concentration. There is evidently a negative exponential salt effect which is Brönsted's prediction for a reaction between two ions of opposite charge. The only assumption in regard to the mechanism that predicts this is that the enzyme is a weak base and that the reaction is between the negative dipeptide ion and the positive enzyme ion. There is probably some doubt, however, as to the application of Brönsted's theory to catalytic reactions and the experiment can hardly be considered conclusive, especially since amines and ampholytes show anomalous changes in activity in the presence of neutral salts (14), particularly those of the type of CaCl_2 .

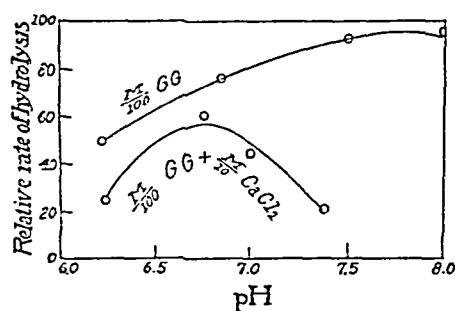


FIG. 5.

FIG. 5. Effect of $M/10$ CaCl_2 on rate of hydrolysis of $M/100$ glycyl glycine at various pH. Erepsin dialyzed in $M/10$ CaCl_2 .

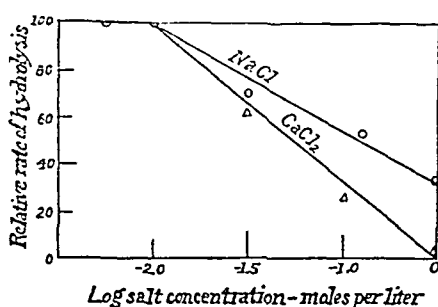


FIG. 6.

FIG. 6. Effect of CaCl_2 and NaCl on hydrolysis $M/100$ glycyl glycine pH 7.0.

Calculation of the Relative Rate of Hydrolysis of Different Substrates.

A number of workers have made determinations of the relative rate of hydrolysis of various peptides by erepsin, *i.e.*, the specificity of the enzyme. These determinations have, as a rule, been made at the same pH. It is evident, however, that if the mechanism assumed in this paper is correct, the determination of the relative rate of hydrolysis of different peptides is a difficult matter, except in the case where the pH-activity curves are the same for the different substrates. In general, however, it would be necessary to correct the observed rate for the fraction of the total enzyme and of the total substrate active at the pH used. The extent of this correction would vary

enormously depending on which of the various possible assumptions are used. In addition it must be shown that the relative rate of hydrolysis of the substrates compared is independent of the concentration. According to the experiments reported in the first part of the paper this is only true in very low concentrations. In view of these considerations it appears impossible, without further information, to make a significant comparison of the rates of hydrolysis of various peptides.

SUMMARY.

1. The rate of hydrolysis at different pH values of glycyl glycine, glycyl leucine, glycyl alanine, glycyl asparagine, glycyl aspartic acid and biuret base has been determined.

2. The pH-activity curves obtained in this way differ for the different substrates.

3. The curves can be satisfactorily predicted by the assumption that erepsin is a weak acid or base with a dissociation constant of $10^{-7.6}$ and that the reaction takes place between a particular ionic species of the enzyme and of the substrate. There are several possible arrangements which will predict the experimental results.

4. The rate of inactivation of erepsin at various pH values has been determined and found to agree with the assumption used above, that the enzyme is a weak acid or base with a dissociation constant of about $10^{-7.6}$.

5. It is pointed out that if the mechanism assumed is correct, the determination of a significant value for the relative rate of hydrolysis of various peptides is a very uncertain procedure.

Most of the experimental work reported in this paper was done by Mr. Frank F. Johnston.

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V

ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life, and appeared simultaneously with Volumes IX, X, and XI.

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THE NATURE OF THE FACTORS WHICH DETERMINE THE SEQUENCE OF GROWTH-CYCLES AND ITS RELATIONSHIP TO THE DIFFERENTIATION OF TISSUES.

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(Accepted for publication, May 25, 1928.)

The Origin of Asymmetry in Growth-Cycles.

In analysing the growth of the white mouse into its component processes¹ it was found that the first and most prolonged growth-cycle, which extends throughout the growing period in these animals, is of the asymmetrical type which is defined by the equation:—

$$\log \frac{x+b}{A-x} = k(t-t_1) \quad (1)$$

where A represents the maximum growth attainable, k the velocity-constant of the growth-process, t_1 is the moment of maximum growth-velocity and b is a constant the essential significance of which is that growth-velocity is already appreciable when x is zero. It also has the effect of rendering the two halves of the cycle on either side of the moment of maximum growth-velocity, unequal in slope and amplitude.

The remaining growth-cycles (the second and third) which are of comparatively brief duration, are of the symmetrical type which is represented by the equation:—

$$\log \frac{x}{A-x} = K(t-t_1) \quad (2)$$

in which the constants have the same significance as above, but growth-velocity is zero when x is zero and the two halves of the growth-cycle, on either side of the moment of maximum growth-velocity, are equal in slope and amplitude.

¹ Robertson, T. B., *J. Gen. Physiol.*, 1925-28, viii, 463.

The applicability of these equations to the growth of mice extends to these animals the rule which has previously been found to obtain in the growth of a variety of other animals² and of plants as well,³ the rule, namely, that the growth of an organism and, frequently, of parts of an organism as well, is an autocatalysed process, by which nothing more is necessarily implied than that *growth is a process of such a nature that it facilitates its own occurrence*. This follows immediately from an inspection of the differential forms of the above equations which may be written:—

$$\text{Symmetrical type:— } \frac{dx}{dt} = kx(A - x) \quad (3)$$

$$\text{Asymmetrical type:— } \frac{dx}{dt} = k(x + b)(a - x) \quad (4)$$

each of which may be regarded as an equation of the monomolecular form, in which the velocity-constant increases in the first case in direct proportion to the product, that is, to the amount of growth achieved, in the second case in proportion to the product (= growth achieved) plus some quantity (= b) which remains unaltered throughout the growth of the animal and has the effect of ensuring that at the beginning of the cycle (which, in the mouse, corresponds with the beginning of gestation) the velocity of growth is considerably greater than that which we would otherwise anticipate from the initial weight of the fertilized ovum.

So far as these facts go, and they may now be considered to have been very thoroughly established in a diversity of forms, they may appear to prove nothing beyond the fact, familiar enough when not formulated in mathematical terms, that growth facilitates its own occurrence. They reveal in addition, however, the fact that this facilitation is quantitative and proportionate to the amount of growing material, and also the fact that growth is limited by some factor (= A) which may either represent some fixed amount of material which

² For literature see Robertson, T. B., *The Chemical basis of growth and senescence*, Monographs in experimental biology, Philadelphia, 1923; also Brody, S. J., *J. Gen. Physiol.*, 1925–28, viii, 233.

³ For literature see West, C., *Sc. Progr.*, 1921–22, xvi, 382.

is consumed during growth or, on the contrary, some accumulation of material which inhibits growth by its mass-action, as a chemical equilibrium is inhibited by the accumulation and mass-action of the products of the forward reaction. The equations reveal nothing whatever regarding the nature of these facilitating and limiting factors in growth which, so far as this information alone is concerned may be physical conditions just as conceivably as they may be chemical substances, provided only that they fulfill the quantitative relation of proportionality to growth achieved which is involved in the applicability of the equations. We are entitled to state, however, as an empirical law of growth, that *the time relations of the growth-process in animals and plants are similar to those which pertain in autocatalysed chemical reactions*. Mere mathematical analysis of the growth-curve, taken by itself, will not lead us further in the interpretation of the actual mechanism of the growth-process. To enable this we must seek additional information of a more directly chemical description. Evidence of this type is afforded by Le Breton and Schaeffer's estimations of the changes in the nucleo-cytoplasmic ratio which accompany the growth of animals.⁴

It has frequently been observed in the growth of plants that the autocatalysis is of the asymmetrical type which is defined by equations (1) and (4).⁵ In animals this asymmetry has been less frequently observed because its effect upon the contour of the growth-curve is greatest at the beginning of growth (when b is large in comparison with x), and the beginning of growth, in the higher animals, is generally inaccessible to accurate measurement because it occurs *in utero*. Nevertheless, one may clearly infer that asymmetry occurs by extrapolating the autocatalytic formulæ of the symmetrical type, which are found to fit the post-natal growth of the animals, to the beginning of gestation. It is then found that the calculated weight at the beginning of gestation is of appreciable magnitude instead of being virtually

⁴ Le Breton, E., and Schaeffer, G., Variations biochimiques du rapport nucleo-plasmatique au cours du développement embryonnaire, Travaux de l'institut de Physiologie, Faculté de Médecine de Strasbourg, Paris, 1923.

⁵ Enriques, P., *Biol. Zentr.*, 1909, xxix, 331. Prescott, J. A., *Sullanic Agric. Soc. Bull.*, No. 13, Cairo, 1924. Brody, S. J., *J. Gen. Physiol.*, 1925-28, viii, 233.

zero⁶ and this magnitude affords an approximate measure of the constant b in the asymmetrical formula which truly defines the growth of the animal. We may therefore conclude that asymmetry of the growth-curve is the rule rather than the exception, and we find, in the mouse at all events, that where the growth-curve in any cycle appears to be symmetrical the cycle is one of very brief duration, covering only a fraction, and that a comparatively late one, of the total growing period.

The asymmetrical equation is equivalent to a symmetrical formula in which the velocity-"constant" varies in proportion to the ratio:—

$$\frac{x + b}{x}$$

which, of course, approaches unity as x (= growth achieved) increases. The asymmetry of the curve of growth implies, therefore, that the velocity-"constant" in the autocatalytic formula does not maintain its initial value but falls off as growth proceeds and in the proportion indicated. The relative values of the velocity-"constants" at various stages of the growth of the animal or plant may therefore be computed from the equation:

$$\frac{k^1}{k} = \frac{x + b}{x}$$

where x is the weight-increment from the moment of fertilization which is attributable to the particular (asymmetrical) cycle under consideration.

If we plot the relative values of k^1 , thus computed, against the total weights of the animals, we obtain a curve which at first falls steeply and later more slowly, approaching the asymptotic value of $k^1 = k$. If, on the same diagram, we plot the relative magnitudes of the corresponding nucleo-cytoplasmic ratios, as estimated chemically by Le Breton and Schaeffer⁷ we find that the two curves are almost precisely parallel in contour, so that, if laid over one another, they almost coincide.⁸ From this we may conclude, without any assump-

⁶ Robertson.¹

⁷ Le Breton and Schaeffer.⁴

⁸ Robertson.¹

tion as to causation, that, as an empirical fact, k^1 is proportional to $\frac{Nc}{Cy}$ where Nc is the mass of nucleic aid contained within the nuclei of all the tissues of the mouse, taken collectively, and Cy is the mass of protein, probably exclusively extra-nuclear.⁹

Although this relationship has as yet been discovered only in the white mouse, because the alteration of nucleo-cytoplasmic ratios with growth has not yet been ascertained in any other organism of which the growth-curve has been accurately defined, yet it is exceedingly improbable that the correspondence of the two curves is accidental, or that any relationship so simple and fundamental is peculiar to one or a few species of animals. It may therefore be justifiable to generalise this result and to state that *the velocity-constant in any autocatalytic cycle of growth is proportional to the nucleo-cytoplasmic ratio*. In what follows we shall assume this rule to be generally applicable and inquire into the nature of the consequences which must necessarily flow therefrom. The fundamental character of these consequences and their close affiliation to the conclusions which are indicated by modern discoveries in genetics, contribute to justify our assumption that proportionality of growth-velocity to the nucleo-cytoplasmic ratio is of general application and not a specific peculiarity of the mouse.

Before proceeding to the consideration of these consequences, however, it must be pointed out that the absence of quantitatively appreciable asymmetry in the later and briefer growth-cycles of the white mouse does not constitute any exception to the rule which we have assumed, because the alteration of the nucleo-cytoplasmic ratio at the relatively late period of growth at which they occur, and for the duration of the periods which these cycles cover, is too small to affect their velocity-constants to a sufficient extent to distort the symmetry of the autocatalytic curve. Such growth-cycles are presumably asymmetrical, but the degree of asymmetry is so small as to fall within the limits of experimental error.

The Sequence of Growth-Cycles.

It has been shown in the communication referred to above¹⁰ that any number of autocatalytic growth-processes, occurring simultaneously

⁹ Abderhalden, E., and Kashiwado, T., *Z. physiol. Chem.*, 1912, lxxxi, 287.

¹⁰ Robertson.¹

within the tissues of an organism, must, *provided that they share a common catalyser*, fuse into a single autocatalytic curve of weight-accretion. "The several processes, in other words, must lose their identity in the combined result."¹¹ The experimental fact, now demonstrated in many species of animals, is that there are two or three distinguishable autocatalytic cycles which contribute to the total growth of the animal without losing their identity in so doing. Only one conclusion can be drawn from this fact, namely, that there exist autocatalytic growth-processes or groups of such processes within the animal and probably, of course, in plants also,¹² which do not share a common catalyser. Such processes may occur within the same cells, or, more probably, in different cells and, in this latter case, we must assume that the several growth-cycles which contribute to the total growth represent the growth of different tissues which share a common catalyser with tissue cells of the same kind, but not with cells of other kinds of which the growth is defined by other and separable autocatalytic curves.

This being the case one would expect to find the velocity constant of autocatalysis varying in proportion to changes in the cells which participate in that cycle, but not, as we have found, in proportion to changes occurring in the tissues of the organism as a whole. If the velocity-constant of autocatalysis were truly dependent upon the nucleo-cytoplasmic ratio, we would expect it to vary in proportion to the change of this ratio occurring in the cells participating in the growth expressed by that cycle, but not to the change in other cells which participate in other cycles. But the estimations of Le Breton and Schaeffer were made upon the whole animal, not upon particular tissues, so that we are driven to infer that the several groups of cells, of which the growth constitutes the different cycles which are distinguishable in the total growth of the animal, nevertheless control or modify each other's growth in some other fashion than by the sharing of a common catalyser.

Only two alternative explanations of this phenomenon appear to be

¹¹ Robertson.¹

¹² Thus the growth of a fruit (Robertson, T. B., *Arch. Entwicklungsmech. Organ.*, 1908, xxv, 581), or seed (Prescott⁵) represents an autocatalytic process clearly distinguishable from the growth-curve of the plant as a whole.

open to us. Either we must assume that tissues which do not participate in a given growth-cycle nevertheless control it by some "action at a distance" or unintelligible "entelechy" which is not the sharing of a common catalyser, or we must assume that the nucleo-cytoplasmic ratios in all the tissues not participating in the first cycle remain stationary until, in consequence of the growth of the cells contributing to the first cycle, their nucleo-cytoplasmic ratios fall to the average value which characterises cells of the second cycle, which then, and not until then, begin to grow, so that the nucleo-cytoplasmic ratios of both groups of cells thereafter fall together in the same proportion. Then, when the cells which contribute the first and second cycles have undergone diminution of their nucleo-cytoplasmic ratios sufficient to bring the ratio down to the level characteristic of the third group of cells, they, in their turn, begin to grow, so that the ratios of all of the cells fall thereafter in proportion to one another.¹³

The ratio $\frac{Nc}{Cy}$ is, in fact:—

$$\frac{Nc_1 + Nc_2 + Nc_3}{Cy_1 + Cy_2 + Cy_3}$$

where Nc_1 , Nc_2 and Nc_3 are the nuclear masses in the cells which participate in the first, second and third growth-cycles respectively, and Cy_1 , Cy_2 and Cy_3 are the corresponding cytoplasmic masses. The hypothesis just outlined indicates a means, and, so far as our knowledge extends, the most probable means of ensuring that the fall of nucleo-cytoplasmic ratios with growth shall be uniform in all the cycles, whether one, two or three cycles are participating in the total growth of the animals during the period under consideration, so that, as we have experimentally ascertained, it must follow that the fall of nucleo-cytoplasmic ratios in the animal as a whole correctly represents the fall in any of the groups of cells which are at the moment participating in the growth of the animal.

¹³ There may, of course, be sundry subsidiary groups of which the growth is inappreciable when merged in the total growth of the animal, so that their growth does not lead to experimentally perceptible fluctuation of the total growth-curve. Such minute subsidiary cycles would be lost to view in the mathematical analysis of the growth of the entire animal.

There are, however, certain implications involved in this hypothesis which merit most careful consideration. Thus, the true nucleocytoplasmic ratio from the beginning of the first cycle to the beginning of the second, is not $\frac{Nc_1}{Cy_1}$ but $\frac{Nc_1 + N}{Cy_1 + C}$, where $N = Nc_2 + Nc_3$ and $C = Cy_2 + Cy_3$. The velocity—"constant" of autocatalysis of growth, during this period, has been found to be proportional to the nucleocytoplasmic ratio *in the animal as a whole*, that is, to $\frac{Nc_1 + N}{Cy_1 + C}$, and this cannot be equal to $\frac{Nc_1}{Cy_1}$ unless $\frac{N}{C}$ is also equal to this ratio, which it could not be unless it varied proportionately with it, that is, unless growth of the second or third groups of cells were occurring, or unless, alternatively, N and C were both so small as to be negligible in comparison with Nc_1 and Cy_1 . Now nearly all of the cells which will ultimately constitute the adult animal have already been produced at birth, and the great majority of them considerably before this.¹⁴ Practically all of the great increase of weight which occurs after birth is due to the increase in the size of cells, only an insignificant fraction of the total growth being attributable, at this stage, to the production of new cells. The data which we are discussing, and the conclusions derived therefrom, apply almost entirely to the growth of pre-existing cells. Clearly, therefore, cells which have not yet undergone growth since their production during embryonic development will constitute, during the earlier stages of growth, a large proportion of the total *number* of cells in the animal, but a very small proportion of the total *weight* of the animal. Hence, since the total growth of the animal prior to the initiation of the second cycle is quantitatively attributable to the first cycle¹⁵ the contribution to the weight of the animal due to cells of the second and third groups must be a negligible proportion of

¹⁴ For example, see Ellis, R. S., *J. Comp. Neurol.*, 1920, xxxii, 1 (Purkinje cells). Macallum, J. B., *Bull. Johns Hopkins Hosp.*, 1898, ix, 208 (Muscle cells).

¹⁵ The "linear increment" which, in mice, is superadded to the growth due to the three autocatalytic cycles, is of inappreciable magnitude at this stage.

the total,¹⁶ so that N and C must, as the second of the above alternatives requires, be negligible in comparison with Nc_1 and Cy_1 .¹⁷

It follows, in other words, that during the period that growth remains as yet solely attributable to the first cycle, $\frac{Nc_1 + Nc_2 + Nc_3}{Cy_1 + Cy_2 + Cy_3}$ remains substantially equal to $\frac{Nc_1}{Cy_1}$ because Nc_2 , Nc_3 , Cy_2 and Cy_3 are, as yet, negligibly small quantities, growth having not yet occurred in the corresponding cell groups. Subsequently to the initiation of the *second* cycle, $\frac{Nc_1 + Nc_2 + Nc_3}{Cy_1 + Cy_2 + Cy_3}$ remains equal to $\frac{Nc_1}{Cy_1}$ because Nc_3 and Cy_3 are, as before, negligibly small quantities, and Nc_2 and Cy_2 , however large they may become, do not begin to increase until $\frac{Nc_1}{Cy_1}$ falls to the initial value of the ratio $\frac{Nc_2}{Cy_2}$ and, however large Nc_2 and Cy_2 may become, they increase in the same proportion to one another as Nc_1 and Cy_1 , so that $\frac{Nc_2}{Cy_2}$, from the beginning of growth due to the second cycle, remains equal to $\frac{Nc_1}{Cy_1}$ and therefore to $\frac{Nc_1 + Nc_2}{Cy_1 + Cy_2}$.

From this it obviously follows that growth due to the second cycle cannot be initiated until the nucleo-cytoplasmic ratio in the cells which participate in the first cycle has fallen to the initial value characteristic of the cells of the second group, that is, the ratios which they possessed when they arose by division. They must remain in a condition of stasis until cells of a higher ratio have fallen to their level, when all can proceed together. Instances of such stasis in tissues or organs for long periods of development, followed by vigorous growth when the development of other tissues has reached the necessary stage are too numerous to require exemplification here. It will be admitted that the above picture of the course of growth and

¹⁶ In the very earliest stages of embryonic development this, of course, will not be strictly true, but with these we are not here concerned. Our data reveal nothing regarding the time and weight relationships in very early embryonic development.

¹⁷ It will, of course, be realized that however small Nc_2 and Cy_2 or Nc_3 and Cy_3 may individually be, their *ratios* may have any magnitude.

development corresponds in this particular very closely with the facts.

From these considerations, therefore, three conclusions emerge, namely:—

1. The cells which participate in the growth composing any autocatalytic growth-cycle have initially lower nucleo-cytoplasmic ratios than the cells which participate in the preceding cycles.

2. Cells of large nucleo-cytoplasmic ratios inhibit growth of cells which possess smaller ratios.¹⁸

3. Subsequently to the production of any cell by division, the increment of cytoplasm always stands in a fixed proportion to the *increment* of nucleus and this proportion is the same for all of the cells of which the organism is composed.

These conclusions offer a very welcome explanation, hitherto lacking, of the relative positions in time of the several autocatalytic cycles which contribute to the total growth of an animal.

In equations (1) and (2) it is easy to comprehend why A , the maximum growth attainable in any cycle, may differ in different cycles. Its magnitude is determined on the one hand by the abundance of available nutrients (the substrates of growth) and, on the other, by the equivalence of these substrates in terms of the products of the growth-process which constitutes that cycle. For since A is measured in terms of the *products* of the growth-process, being their final mass, if the products be such as are expensive to produce, either on account of the high velocity of the reverse reaction (breakdown of tissue elements when produced) or because of the large number of molecules of substrate required to participate in their formation,¹⁹ then A for that

¹⁸ Possibly by prior appropriation of nutrients, due to their higher metabolic rate (Robertson²).

¹⁹ This would be the case, for example, in the synthesis of connective-tissue proteins which are of abnormal amino-acid composition, so that many molecules of protein of average composition might have to be decomposed into their constituent amino-acids to produce those required for the manufacture of a single molecule of the aberrant protein.

cycle will be small in comparison with its value in a cycle in which the products are less expensive to manufacture.²⁰

Similarly, we can understand why k should vary in different cycles, because the reactants in each cycle differ from those in any other. But it is not immediately obvious what physico-chemical mechanisms determine the magnitudes of t_1 , t_2 and t_3 , the moments, namely, at which the autocatalytic growth-cycles attain their maximum velocities. These are clearly related to the moments at which the growth due to each cycle becomes of appreciable magnitude. Given the values of k , A and t_1 , t_2 or t_3 for any cycle, it is possible to compute the time (that is, age) at which the weight-increments due to that cycle must become of measurable magnitude. The value of t_1 , however, is simply computed, in practice, from the observations. Its value has not hitherto been predictable from any other consideration, nor has it been possible to refer it to any determinative factors. The hypothesis outlined above establishes such factors and enables us to understand why the onset of later cycles is necessarily delayed until the preceding cycles have attained a certain stage of development.

The Relationship of Growth-Cycles to Differentiation.

The conclusions reached above, and derived from a comparison of the time-relations of growth with the corresponding changes of nucleocytoplasmic ratio, fit with remarkable precision into the hypothesis of differentiation which the author has put forward elsewhere, basing it upon data of totally different origin and description.²¹

It is a well-known fact that the early cleavage-cells produced at the beginning of the development of an embryo, are toti-potent, that is, they are individually capable, even in the absence of the other cleavage-cells, of giving rise to the complete embryo. At a comparatively early stage of development, however, usually within a few cell-divisions, this toti-potency is lost and becomes replaced by a partial capacity for

²⁰ The proportion of water incorporated within the living tissue would also constitute an important factor modifying the value of A . Attention has been drawn to this fact in communications by Cramer, W., and Pringle, H., *Proc. Roy. Soc. London, Series B.*, 1910, lxxii, 307, 315.

²¹ Robertson.²

reproduction capable of replacing large portions of the embryo which may have been lost through mutilation. Progressively the reproductive potency of the cells of the embryo diminishes until, in the adult, it remains only to the extent that tissues possess the power of repairing injury to themselves.

The most natural interpretation of these facts, in the light of the chromosomal theory of inheritance, is that the chromosomal heritage of cells produced in later stages of development is incomplete, so that they retain only a corresponding fraction of the original power, possessed by the fertilised ovum, of reproducing the whole organism. If, however, the chromosomal endowment of the more recent cells is less than that which the parent cells initially possessed, this can only be because division occurred before the nuclear substance of the parent-cell had been completely reduplicated.²² According to this hypothesis, therefore, differentiation, in the development of an organism, is attributable to the achievement by certain cells of the power of undergoing division at a lower nucleo-cytoplasmic ratio than that at which the parent-cells divided. Such cells must possess a different physiological character from their parents and react, in consequence, in a different manner to their environment. The reaction to the environment determines the type (*i.e.* structure and composition) of cytoplasm which is produced, so that from the altering nucleo-cytoplasmic ratios of successive generations of cells spring altering physiological responses and consequent differentiations of structure.

Obviously, if this were the case we would expect to find, at the conclusion of cell-production in development, various groups of cells which differ in their nucleo-cytoplasmic ratios, those of latest production being possessed of the lower ratios. The conclusions arrived at in this paper, and based upon quite independent evidence, also point to the existence, within the developing animal, of groups of cells which are distinguishable from one another by the possession of different nucleo-

²² It must be recollected that the element of the nucleus which stains with hematoxylin is nucleic acid (Mathew, A. P., *Am. J. Physiol.*, 1898, i, 445). The chromosomes to which the geneticist traces the inheritable characters of the adult are therefore masses of nucleic acid, whatever else may chance to be associated with them.

cytoplasmic ratios. Our conclusions drawn from the study of growth itself and chemical estimations of the nucleo-cytoplasmic ratio contribute, therefore, very materially, to support the above theory of differentiation.

The Relation of the Nucleus to the Growth of Cytoplasm.

Confining our attention to a single asymmetrical cycle of growth, such as the first growth-cycle in the mouse, since we have found that the velocity constant of growth is given by the proportion:—

$$\frac{k^1}{k} = \frac{x + b}{x}$$

and we have also found that $\frac{k^1}{k}$ is proportional to $\frac{Nc}{Cy}$, that is, that $\frac{k^1}{k} = \rho \frac{Nc}{Cy}$ where ρ is a constant proportionality-factor, it follows that:—

$$\frac{x + b}{x} = \rho \frac{Nc}{Cy}$$

Now x , the weight of an animal is almost entirely²³ the weight of its cytoplasmic constituents and products. We may, therefore, with very close approximation to the truth, write Cy for x in the above equation, from which it follows that:—

$$Cy + b = \rho Nc$$

and hence that:—

$$\rho \frac{dNc}{dt} = \frac{dCy}{dt}$$

or, in other phraseology, that the growth of cytoplasm, subsequent to the production of the cell by the cell-division from which it arose, stands always in a fixed proportion to the growth of the nucleus, a conclusion to which we have already been impelled for other reasons.

²³ That is, over 90 per cent. In thymus, which represents a comparatively undifferentiated and richly nucleated type of tissue, the total nuclear constituents, chromosomal and others, constitute 15 per cent of the weight of the gland.

It also follows that, substituting $Cy + b$ for $x + b$ in equation (4):—

$$\frac{dx}{dt} = k \rho Nc(A - x)$$

in which k and ρ are constants and A is the maximum growth attainable in the cycle. From this we derive the very important and suggestive conclusion *that the growth of cytoplasm is determined by a monomolecular process which is catalysed by the nucleus in proportion to its mass.* The autocatalytic character of the growth-process in animals and plants takes its origin, therefore, in the fundamental dependence of cellular syntheses upon the presence of the nucleus or its products.

The error involved in assuming the identity of x (total growth achieved) with Cy (the mass of cytoplasmic material) is very small and diminishes, of course, as the nucleo-cytoplasmic ratio decreases.²⁴ The simple and fundamental character of the relationship deduced by making this approximation suggests, however, that this represents the true relationship and that the empirical formula:—

$$\frac{dx}{dt} = k(x + b)(A - x)$$

represents, in fact, a mathematical approximation. We may, in consequence, expect to find, as we approach the beginnings of embryonic development (when $\frac{Nc}{Cy}$ is at its maximum value and the error involved in neglecting Nc in comparison with Cy is therefore at its maximum also) that the autocatalytic equations, as hitherto formulated, no longer apply. The necessary measurements of very early embryonic

²⁴ If the chromosomal constituents of the nuclei were to represent as much as 10 per cent of the total weight of the organism, the departure from proportionality of nuclear to cytoplasmic increment would only be 1 per cent, for in equalising $\frac{Nc}{Cy}$ to $\frac{Cy + b}{Cy}$ instead of to $\frac{Cy + Nc + b}{Cy + Nc}$ only the *square* of the nuclear mass need be neglected to ensure proportionality of the nuclear and cytoplasmic increments. The actual error, during the period of development covered by post-natal growth-curves, is probably much less than this and therefore far within the magnitude of the experimental error of weight determinations in animals.

growth have not yet been carried out, however, with sufficient frequency and exactitude to permit the demonstration of these expected deviations from the autocatalytic formulæ.

The tendency of all of these results, therefore, is to indicate that the nucleus plays a predominant rôle in determining the growth of cytoplasm. This conclusion emerges, be it noted, solely from the quantitative analysis of the growth-process itself, coupled with the empirical correspondence between growth-velocity and nucleo-cytoplasmic ratios. This being the case it is a most noteworthy fact that the conclusions thus derived stand in such remarkable accord with the indications of modern genetic research. From the study of the time-relations of growth and the concurrent alterations of nucleic acid content of the animal, on the one hand, and from the study of cytology and inheritance upon the other, we attain to the same conception of the nucleus as the predominating factor in shaping the development of the cell in which it resides.

CONCLUSIONS.

1. It has previously been shown by the author and many others that growth, in animals and plants, is an autocatalysed process. In animals it is usual to find that growth occurs in several superimposed autocatalytic cycles. In many cases, in plants and animals, especially if the cycle is one which occupies a large proportion of the growing period, it is found that the velocity-constant of the autocatalysed monomolecular formula falls off as growth proceeds, at first rapidly and later more slowly.

2. It has previously been shown by the author that the fall of the velocity-constant of growth, in the white mouse, is directly proportional to the fall of the nucleo-cytoplasmic ratio, determined by the chemical method of Le Breton and Schaeffer. If we assume this relationship to be generally applicable to the growth of animals and plants, then the following additional conclusions may be deduced, without calling in the aid of any other assumption:—

3. The increase of cytoplasm in any given cycle of growth is proportional to the concurrent increase of nuclear material.

4. The growth of cytoplasm takes place in accordance with a monomolecular formula in which the velocity-constant varies directly as the mass of the nucleus.

If we superadd to these facts and deductions the hypothesis that each growth-cycle represents the growth of a separate group of cells within the animal, then the additional conclusions follow:—

5. That the cells which participate in the growth composing any cycle have initially lower nucleo-cytoplasmic ratios than the cells which participated in the preceding cycles.

6. That cells of large nucleo-cytoplasmic ratios in a multicellular animal inhibit the growth of cells which possess smaller ratios.

7. These conclusions collectively imply that the nucleus plays a predominant rôle in determining the development of the cell in which it resides.

THE THERMIC EFFECT OF DEATH.

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The principal chemical compounds which compose living matter (aside from water) presumably consist largely of protein substances and lipoids and are very unstable. As explosive substances they can be destroyed not only by chemical and physical but even by purely mechanical agents. A complete decomposition of these compounds brings about death.¹ As the decomposition of very unstable chemical compounds is usually accompanied by the production of heat, we may expect the decomposition of the principal compounds of living matter and death to be accompanied by the production of heat.

In this paper I intend to describe the results of my experiments on the thermic effect of death.

Material and Method.

In my experiments I used yeast which was mixed with distilled water. As it had only little oxygen and no sugar at its disposal its respiratory processes were inhibited and no production of heat could be observed if the mixture of yeast and water was placed in the calorimeter. The yeast used was Fleischmann's compressed yeast washed with distilled water. The calorimeter used was a Dewar flask closed by a rubber stopper through one of two openings of which a Beckmann thermometer was inserted. The second opening was made for the introduction of poisons into the flask. The flask was placed into a wooden box filled with shavings.

The Dewar flask was first used as a calorimeter by Rubner² who determined the heat produced by alcoholic fermentation and by the inversion of sugar. The volume of the flask was somewhat greater than that used by Rubner but the

¹ Lepeschkin, W. W., *Biol. Zentr.*, 1926, xlv, 487; *Kolloidchemie des Protoplasmas*, Berlin, 1924, 155; *Arch. exp. Zellforsch.*, 1927, iv, 212; *Protoplasma*, 1927, ii, 239.

² Rubner, M., *Arch. Hyg.*, 1904, xlviii, 274; xlix, 335, 398.

walls were the same (the volume was 450 cc.). In the experiments described by Rubner, heat was produced very slowly, and the loss of heat by the flask, during the experiment, had to be taken into account. In my experiments the production of heat by death continued, however, for a very short time, not more than a few minutes. The loss of heat from the flask during the period of the experiment was therefore so inconsiderable that it could be disregarded.

I should like to cite here the results of my experiments concerning the constancy of temperature in the Dewar flask filled with water.

The position of mercury in the Beckmann thermometer was such that the temperature 2.300° corresponded to 20.9°C . The changes of temperature in the flask are given in the following table.

Date	Time	Temperature in the flask (Beckmann)	Temperature in the room (Centigrade)
October 13	12:30 p.m.	2.300	20.9
	3:00 p.m.	2.302	22.2
	4:00 p.m.	2.310	21.1
October 14	7:15 a.m.	2.290	20.3
	8:45 a.m.	2.293	22.8
	11:15 a.m.	2.303	22.6
	12:45 p.m.	2.321	22.8

From the cited results we may conclude that the change of temperature in the room produces no considerable effect upon the temperature in the flask. The greatest change was 0.031°C . and it occurred only after an interval of $5\frac{1}{2}$ hours with the room temperature increased from 20.3° to 22.8°C . Since the temperature in the flask, during the experiment, did not exceed the room temperature more than 0.5°C . we may assume that it remained constant during the experiment which lasted only 5 or 10 minutes.

On the other hand in order to kill yeast cells I had to mix the poison with the yeast suspension, by stirring the liquid in the flask. It was therefore necessary to know whether the stirring changes the temperature of water in the flask. This stirring was produced, in my experiments, by an energetic shaking of the flask.

Temperature in the flask (Beckmann)	Temperature after the first stirring	Temperature after the second stirring	Temperature after the third stirring
2.362	2.372	2.377	2.378
1.992	2.015	2.022	2.022
1.723	1.755	1.762	1.763
2.015	2.011	2.010	2.010

From the above experiment it is apparent that before the introducing of poisons the liquid must be shaken until the temperature of the liquid ceases to

change. Then we can be certain that the temperature will not be altered by mixing poisons with the liquid in the flask.

The death of yeast was produced, in my experiments, by adding either, corrosive sublimate or chloroform. In order to make the temperature of the poisons exactly equal to the temperature of the liquid in the flask the poisons were introduced into the flask in a special glass tube which remained there for about 1 hour before mixing the poisons with the liquid. This time is sufficient for an equalization of temperature of the poison and the liquid in the flask. The tube used (about $1\frac{1}{2}$ cm. in diameter, 7 cm. long, volume $6\frac{1}{2}$ cc.) was drawn out at the lower end to the diameter of 2 mm. and bent upwards (Fig. 1). The upper end of the tube was provided with a long rubber tube closed by means of a pinch-cock and inserted through the second opening of the rubber stopper of the flask. The tube was filled with a saturated solution of corrosive sublimate or with chloroform of the same temperature as the suspension in the flask. The volume of each was exactly $5\frac{1}{2}$ cc. The poisons were introduced into the liquid by blowing into the rubber tube after opening the pinch-cock. A special experiment showed that such blowing did not alter the temperature in the flask.



FIG. 1

The action of sublimate on protoplasm has peculiarities which must be considered in the determination of the thermic effect of death. Sublimate alters living matter chemically producing salt like compounds with proteins. It is very probable that the formation of such compounds is accompanied by the production of heat, and this heat must be subtracted from the heat produced by the action of corrosive sublimate on yeast. The heat of the formation of compounds between sublimate and the proteins of yeast could be determined on the following basis.

Mercury bichloride acts on proteins of all kinds in the same manner. Hydrogen of the carboxyl groups of proteins is replaced by mercury, so that a salt-like compound arises in all cases. On the other hand, as is known, the formation heats of salts of different organic acids which possess an analogous structure (with the same metal) are approximately equal.³ The formation heats of salt-like compounds of mercury with different proteins are therefore alike. Thus in order to determine the heat produced in the reaction between sublimate and the proteins of yeast we can limit ourselves to a determination of

³ Berthelot, M., *Ann. chim. et phys.*, 1875, vi, series 5, 326. Massol, G. 1894, i, series 7, 145.

heat of the reaction between sublimate and any common protein, as for instance, albumin. Both heats must be approximately equal.

The Thermic Effect Produced in the Reaction between Corrosive Sublimate and Albumin of Eggs.

40 gm. albumin of eggs (Merck) were dissolved in 400 gm. distilled water, and the solution (volume 425 cc.) was poured into the Dewar flask. Then the glass tube (Fig. 1) filled with a saturated solution of sublimate was introduced into the flask, and the latter was closed with the rubber stopper. In 1 hour the temperature in the flask was 1.723. After an energetic stirring the temperature increased to 1.750; after the second stirring it was 1.762 and after the third stirring it was 1.767; further stirring did not alter this temperature. Immediately the sublimate solution was blown out of the tube into the albumin solution, and the mixture was stirred. The temperature increased at once to 1.796. The further stirring raised the temperature to 1.820, but it at once began to decrease. Therefore we can conclude that the heat produced in the reaction between sublimate and albumin warmed the liquid in the flask 0.053°C . At the same time it warmed the thermometer and the flask itself.

The specific heat (heat capacity) of the albumin solution (10 per cent) is about 0.9; as to 425 cc. of this solution 5.5 cc. solution of mercury chloride was added and as the specific heat of diluted solutions of mercury chloride is almost equal to that of water the heat which is necessary to warm the liquid in the flask 0.053°C . is $430 \times 0.9 \times 0.053 = 20.5$ gm. calories. The Beckmann thermometer contained about 40 gm. mercury, and it therefore absorbed about $40 \times 0.03 \times 0.053 = 0.06$ gm. calories. Concerning the heat absorbed by the flask my experiments showed, in accordance with Rubner² (page 372), that it is about $15 \times 0.053 = 0.79$ gm. calories. The total heat produced in the reaction was about 21 gm. calories or per gram dry albumin $21 \div 40 = 0.52$ gm. calories.

We shall now describe the results of the determination of the heat produced by the death of yeast.

The Thermic Effect of Death Produced by Corrosive Sublimate.

82.44 gm. yeast (washed and freed from water by pressure) were mixed with 400 gm. distilled water. The mixture was stirred until all yeast cells were separated from each other. Then it was poured into the Dewar flask. The yeast contained 64 per cent water and, therefore the dry substance of the yeast used was 29.68 gm. After stirring the liquid several times the final temperature was 4.014° , that is 22.6°C . Soon after the glass tube (Fig. 1) filled with a satu-

rated solution of sublimate of the same temperature was introduced into the flask and the flask was closed by the rubber stopper.

In 1 hour the liquid in the flask was stirred again several times, and the final temperature was 4.017. Immediately the solution of mercury chloride is blown out from the glass tube into the suspension of yeast, and the liquid again stirred. The temperature of the liquid increased at once to 4.090, the maximal temperature.

The microscopical investigation of the yeast taken from the flask showed that only 50 per cent of the cells were killed by sublimate (test by methylene blue). The size of the Dewar flask did not permit, however, the use of a greater quantity of mercury chloride. The tube used contained only 5.5 cc. of solution.

From the cited results we can conclude that the heat produced by the action of sublimate on yeast warmed the liquid in the flask, the thermometer and the flask itself 0.073°C. According to Rubner² (page 372) the specific heat of yeast is 0.785. Therefore the heat produced by the action of corrosive sublimate on the yeast is: $0.073 (405 + 82.4 \times 0.785 + 40 \times 0.03 + 15) = 35.4$ gm. calories or per gram of dry yeast $35.4 \div 29.68 = 1.19$ gm. calories. Subtracting from this value the heat produced in the reaction between mercury chloride and protein substances of the yeast (dry yeast contains about 50 per cent protein substances⁴ and only 50 per cent of the cells were killed) that is $0.52 \times 0.5 \times 0.5 = 0.13$ gm. calories we reach the conclusion that there was an additional effect amounting to $1.19 - 0.13 = 1.07$ gm. calories per gram of dry yeast, or about 2 gm. calories per gram of the dry substance of killed yeast (50 per cent of the cells only were killed).

The Thermic Effect of Death Produced by Chloroform.

As is known chloroform does not form chemical compounds with protein substances, but it is taken up by protoplasm and strongly accelerates the denaturation of protein substances. Such denaturation may bring about death.

80 gm. yeast which contained 24.8 dry substance were mixed with 400 gm. distilled water, and the mixture was stirred until all cells

⁴ König, J., *Chemie der menschlichen Nahrungs- und Genussmittel*, Berlin, 1919-23, 1056.

were separated from each other. Then the mixture was poured into the Dewar flask. The temperature in the flask was, after stirring several times, 0.825° (Beckmann). The glass tube (Fig. 1) containing 5 gm. chloroform was then introduced into the flask, but the chloroform was not blown out of the tube.

In 1 hour the liquid in the flask was stirred again several times, and the final temperature was 0.875° . Immediately chloroform was blown out from the glass tube into the suspension of yeast, and the mixture again stirred. The temperature rose at once to 0.910 , and this temperature was not altered by the further stirring of the mixture. The temperature rise is therefore 0.035°C .

We can therefore conclude that the death of yeast was accompanied by the production of heat of $0.035 (400 + 5.5 \times 0.23 + 80 \times 0.785 + 1.2 + 15) = 16.7$ gm. calories, or per gram of killed yeast $16.7 \div 24.8 = 0.67$ gm. calories. As the microscopical investigation showed that only 50 per cent of the yeast cells were killed this heat per gram of killed yeast is about 1.5 gm. calories. The obtained value is somewhat smaller than that obtained in the former experiment, but the difference does not exceed the experimental error.

The Action of Corrosive Sublimate and Chloroform on Dead Yeast.

Up till now we considered the thermic effect produced by the action of mercury bichloride and chloroform on living yeast as a consequence of death, that is, of the decomposition of the principal chemical compounds composing living matter. In the case of bichloride this effect is, at the same time, a consequence of the reaction between bichloride and proteins of yeast; but the latter thermic effect proved to be small in comparison with the effect produced by death.

One could suppose however that the thermic effect which we ascribe to death could perhaps be produced by other unknown processes in which both poisons possibly play a part, as for instance, by an adsorption of them by the substances of yeast and so on. I should like therefore to show here that the thermic effect observed is produced by death itself. Indeed, if the same manipulations are repeated on dead yeast we either observe no thermic effect or this effect corresponds only to the reaction between mercury bichloride and proteins of yeast.

80 gm. yeast (washed and freed from water by pressure) were mixed with 400 gm. distilled water. The mixture was stirred until all yeast cells were separated from each other. Then it was heated to 80°C. so that all yeast cells were killed. After the suspension took the temperature of the experimental room it was poured into the Dewar flask. The yeast contained 25.5 gm. dry substance.

After stirring the liquid several times the final temperature was 2.395° (Beckmann). Soon after the glass tube (Fig. 1) filled with a saturated solution of corrosive sublimate of the same temperature was introduced into the flask and the flask was closed by the rubber stopper.

In 1 hour the liquid in the flask was stirred again several times, and the final temperature was 2.417°. Immediately the solution of sublimate (5.5 cc.) was blown out of the glass tube into the suspension of dead yeast, and the liquid again stirred. The temperature of the liquid increased at once to 2.423°. The further stirring did not alter this temperature.

From the cited results we can conclude that the heat produced by the action of sublimate on dead yeast warmed the liquid in the flask, the thermometer, and the flask itself 0.006°C. Therefore the heat produced was $0.006 (400 + 82.4 \times 0.785 + 20 \times 0.3 + 15) = 3$ gm. calories or per gram of dry yeast $3 \div 25.5 = 0.12$ gm. calories. This heat corresponds to the heat produced in the reaction between albumin and mercury bichloride (0.13 gm. calories).

No effect of chloroform on dead yeast could be observed. The experiment was made in the same manner as that with mercury bichloride. The final temperature in the Dewar flask containing a tube filled with 5.5 cc. chloroform was 2.479°. After mixing chloroform with the suspension of yeast and stirring several times it did not change at all.

The Thermic Effect of Death as Compared with Heat Produced in Other Chemical Reactions.

The thermic effect of death observed in my experiments is, of course, very small as compared with heat produced in most of the

chemical exothermic reactions. The heat produced by the decomposition of HI to H and I is accompanied, for instance, by the production of 46 gm. calories per gram, and the decomposition of N_6Hg_2 gives 290 gm. calories per gram.

If we consider however the molecular weight of the principal compounds of living matter as very great, we come to the conclusion that the heat produced by the decomposition of 1 molecule of these compounds is of the value which could be compared only with values observed on explosives. Indeed the principal compounds of living matter contain proteins as a component part, but the molecular weight of the latter is very great, as for instance, the molecular weight of hemoglobin (determined in the chemical analysis according to the content of iron) is 16,669. The molecular weight of the principal chemical compounds of living matter is certainly still greater. Thus if the molecular weight of these compounds had been even like that of hemoglobin the heat produced by the decomposition of 1 gm. molecule of these compounds would at least be $3 \times 16,669$,* that is greater than 40 kilo calories, while the heat produced by the destruction of 1 gm. molecule of HI is only 6 kilo calories, that of NCl_3 is 38 kilo calories, and so on. Therefore, on the average, the heat produced by the decomposition of 1 gm. molecule of the principal compounds of living matter is more than that of such an unstable and explosive body as NCl_3 . Thus it is very comprehensible that the molecule of the principal chemical compounds composing living matter can be destroyed not only by chemical and physical but also by purely mechanical agents. This molecule is so unstable as to be compared with the molecules of explosive substances.

SUMMARY.

The thermic effect of death as observed on yeast is about 2 gm. calories per gram of dry substance and is therefore very small as

* The thermic effect of death is, on the average, 1.7 gm. calories per 1 gram of dry yeast. If we suppose that even all protein substances (50 per cent) and lipoids (5 per cent) form the principal compounds (aside from water) of its living matter we must conclude that the thermic effect of death is $1.7:0.55 = 3.1$ gm. calories per gram of these compounds.

compared with the heat produced in most of the exotherm chemical reactions. As the chemical compounds composing living matter have a very great molecular weight, the decomposition of their molecule which brings about death produces more heat than the decomposition of the molecule of some explosive substances. This explains the great instability of the principal compounds of living matter.

Addendum.—After the correction of the proof my attention was drawn to two papers concerning the problem discussed above. In one of these papers Peters describes his experiment in which muscles were poisoned with chloroform.⁵ The heat produced hereby (calculated per gram of dry substance) is approximately equal to the heat observed in my experiments on yeast. Muscles can be considered as consisting of living matter, and therefore Peters' results are in complete accord with my results. In the other paper Meyerhof tried to determine the heat produced by the action of acroleine on erythrocytes;⁶ the poisoning was extended until the respiration irreversibly ceased. Meyerhof admitted that he observed the death of erythrocytes, though, according to his description, no hemolysis was observed; indeed Meyerhof writes: "Wird dasselbe in genügend hoher Konzentration zu lebenden Zellen zugesetzt, so tritt eine totale Atmungs-hemmung ein, die bei einem wenige Minuten später stattfindenden Wegwaschen des Giftes nicht mehr zurückgeht." A washing out of poison can certainly only be performed if no hemolysis occurs. It is possible that the heat produced by the death of erythrocytes is smaller than 1.4 gm. calories, as Meyerhof admits (1.4 calories is the limit of the exactness of his experiments), but it is more probable that the erythrocytes were not dead in Meyerhof's experiments, because the death of erythrocytes brought about by acroleine must be accompanied by hemolysis.

⁵ Peters, R. A., *J. Physiol.*, 1913-14, xlvii, 262.

⁶ Meyerhof, O., *Arch. ges. Physiol.*, 1912, cxlvi, 181.

THE DEATH WAVE IN NITELLA.

II. APPLICATIONS OF UNLIKE SOLUTIONS.

By W. J. V. OSTERHOUT AND E. S. HARRIS.

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(Accepted for publication, June 27, 1928.)

Previous experiments lead us to expect that protoplasm will give a negative current of injury when in contact with dilute solutions and that the opposite will be true with concentrated solutions. This should enable us to predict what will happen when a cell is brought simultaneously in contact with both kinds of solutions. It is shown in this paper that such prediction is possible.

These predictions involve the assumption that the protoplasm is composed of layers, *i.e.* an outer (*X*) and an inner (*Y*) both of which are probably non-aqueous and between these an aqueous layer (*W*). As previously explained¹ the more rapid alteration of the outer layer produces a positive current of injury and the more rapid alteration of the inner gives a negative current of injury. The latter is observed when the protoplasm is in contact externally with a solution less "effective"² than sap (*e.g.* 0.001 M KCl); the former occurs with solutions more effective than sap (*e.g.* 0.1 M KCl.) Hence if we apply 0.001 M KCl to the cell the outer layer (*X*) will be in contact with a less effective solution than the inner (*Y*) (which is in contact with sap).

If we arrange an experiment as in Fig. 1 with 0.001 M KCl at *A* and 0.1 M KCl at *C*,³ and cut at *Z* we shall expect that, as previously described,¹ the death wave which starts at *Z* will pass rapidly down the cell to *C*. At *A*, where the protoplasm is in contact with 0.001 M KCl, we shall expect the inner layer (*Y*) to go first so that the protoplasm will at first become more negative¹ but at *C*, where it is in contact with 0.1 M

¹ Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, xii, 167.

² The more effective solution is the one which causes a more rapid alteration.

³ The technique has been fully described in previous papers. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 391, 417, 673; 1928-29, xii, 167.

KCl, the alteration of X will be more rapid than that of Y and in consequence the protoplasm will first become more positive.

That this is really so can be shown by arranging an experiment as in Fig. 2, killing B with 0.01 M KCl saturated with chloroform and leading off from A (in contact with 0.001 M KCl) to B and from C (in contact with 0.1 M KCl) to B : on cutting at Z we obtain approximately the "true" curves of A and C (*i.e.* the curves obtained when the value at B is zero), as shown in Fig. 3 *a*.⁴ If we lead off from A to C we obtain

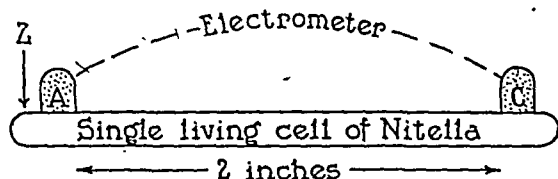


FIG. 1. Diagram to show the arrangement of certain experiments. At A and C are flowing contacts, or pieces of cotton soaked in solutions, or cups containing solution.

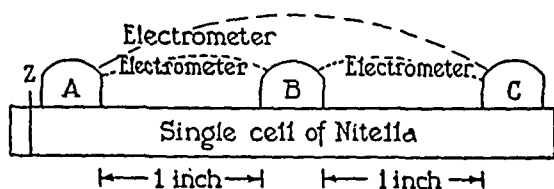


FIG. 2. As in Fig. 1 with an additional contact at B which is connected with A and with C .

approximately the difference between the "true" curves of A and C , as shown in Fig. 3 *b*.

The result corresponds to our prediction, *i.e.* at A (which is in con-

⁴ The values at A will be more positive than they should be by a small but definite amount because of an effect due to the cell wall (see Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 673) which makes A appear more positive. This effect will remain constant until sap diffusing out at B raises the concentration of the solution imbibing the cell wall; the effect of this will be to make A appear still more positive but in very short experiments it may be neglected. What is said of A applies equally to C (in contact with 0.1 M KCl) except that the cell wall effect will make C appear somewhat more negative than it should be at the start and the coming out of sap at B will decrease this effect. These effects are small and may be neglected for our present purpose.

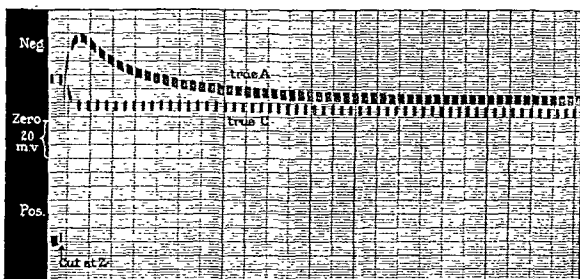


FIG. 3 a.

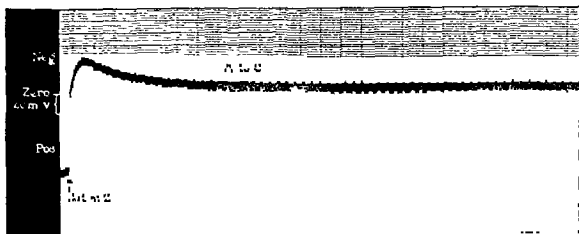


FIG. 3 b.

FIGS. 3 a and b. Photographic records showing potential differences, the experiment being arranged as in Fig. 2 with 0.001 M KCl at A, 0.01 M KCl at B, and 0.1 M KCl at C. The record starts after B has been killed (with 0.01 M KCl saturated with chloroform) so that A and C show their "true" values. On cutting at Z we see (in Fig. 3 a) that A becomes negative and then approaches zero while C becomes positive and then approaches zero. Curves A and C are recorded on one instrument while the A to C curve is simultaneously recorded on another (Fig. 3 b): this curve is approximately equal to the difference between the "true" curves of A and C.

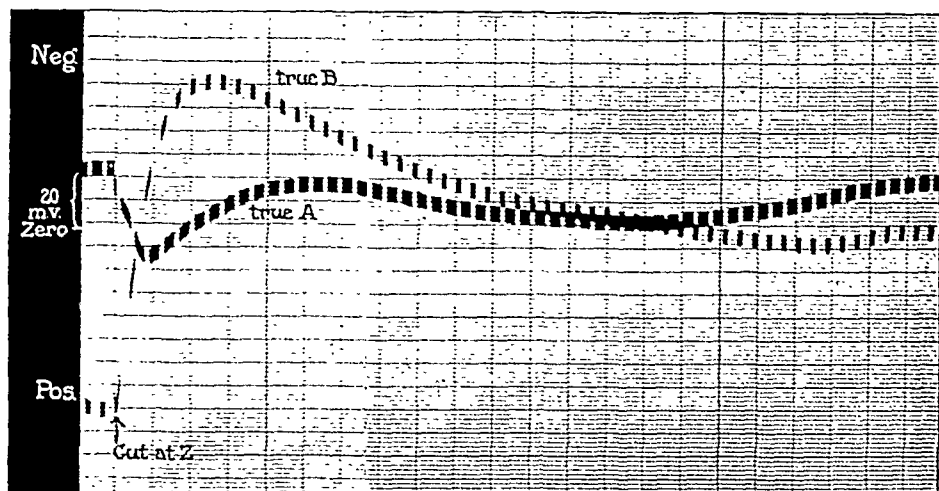


FIG. 4 a.

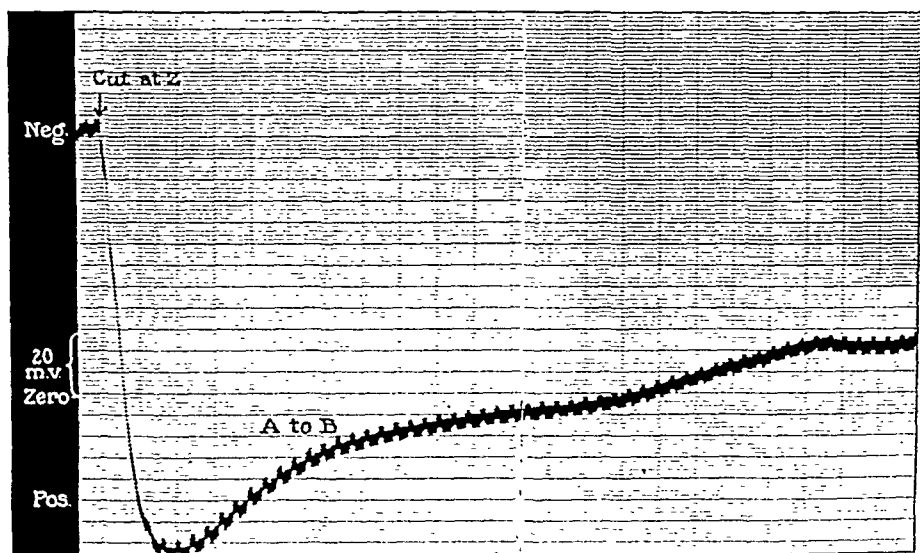


FIG. 4 b.

FIGS. 4 a and b. Photographic records showing potential differences, the experiment being arranged as in Fig. 2 with 0.1 M KCl at A, 0.001 M KCl at B, and 0.01 M KCl at C. The record starts after C has been killed (with 0.01 M KCl saturated with chloroform) so that A and B show their "true" values. On cutting at Z we see (in Fig. 4 a) that A becomes positive and then approaches zero while B becomes negative and then approaches zero. Curves A and B are recorded on one instrument while the A to B curve is simultaneously recorded on another (Fig. 4 b): this curve is approximately equal to the difference between the "true" curves of A and B.

tact with a solution less effective than sap) the protoplasm first becomes more negative as the result of the cut, indicating that the layer I' goes first; at C (which is in contact with a solution more effective than sap) the protoplasm first becomes more positive, indicating that the layer X goes first.

It is desirable to supplement this experiment by placing 0.1 M KCl at A , 0.001 M KCl at B , and then killing C : the result is shown in

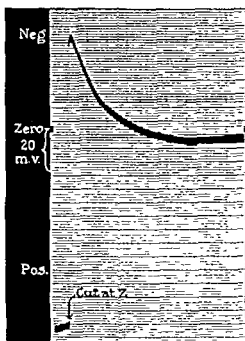


FIG. 5.

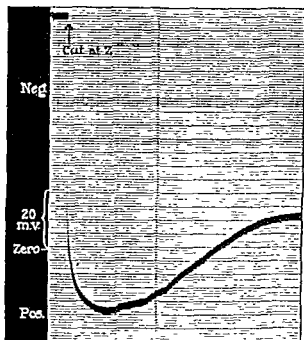


FIG. 6.

FIG. 5. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.001 M KCl at A and 0.1 M KCl at C . On cutting at Z we obtain a curve resembling that in Fig. 3 *b*.

FIG. 6. Photographic record of potential differences, the experiment being arranged as in Fig. 1 with 0.1 M KCl at A and 0.001 M KCl at C . On cutting at Z we obtain a curve similar to that in Fig. 4 *b*.

Figs. 4 *a* and 4 *b* (since C is killed we obtain the "true" curves of A and B). The result is again in accordance with prediction.

It may be thought that killing a spot may alter the condition of the cell and so affect the curves. We have accordingly performed experiments, (arranged as in Fig. 1) in which the killing is omitted: these are illustrated by Figs. 5 and 6 which resemble Figs. 3 *b* and 4 *b* sufficiently to indicate that the killing of a single spot makes no essential

difference. In all these cases we are dealing with the difference between two spots, *A* and *C*, or *A* and *B*, which are affected in turn by a death wave which starts from *Z* and travels down the cell.

An interesting experiment is shown in Fig. 8. The experiment was arranged as in Fig. 7 with 0.1 M KCl at *A*, 0.001 M KCl at *B*, and 0.05 M KCl at *D* and at *C*.

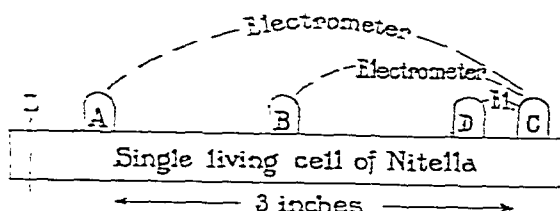


FIG. 7. As in Fig. 1 with additional contacts at *B* and *D*.

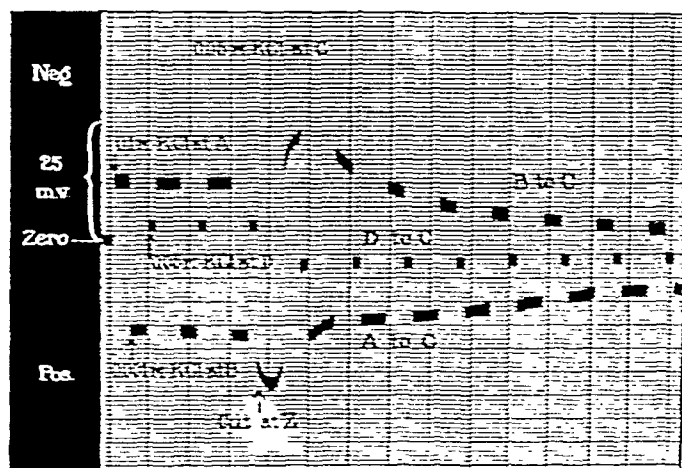


FIG. 8. Photographic record of potential differences, the experiment being arranged as in Fig. 7 with 0.1 M KCl at *A*, 0.001 M KCl at *B*, 0.05 M KCl at *D* and at *C*. On cutting at *Z* we see that *A* becomes positive and then approaches zero, *B* becomes negative and then approaches zero; *D* shows little change because it is in this case very near *C* and is in contact with the same solution: it becomes slightly positive (as expected) and then approaches zero.

M KCl at *D* and at *C*. At the start *A* is negative and *B* positive due to the concentration effect.⁵ On cutting at *Z*, *A* changes from negative to positive and *B* from positive to negative. *D* shows little change

⁵ This is discussed in Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 673.

because it is very close to C and is in contact with the same solution (its curve is in accordance with expectation¹).

The fact that B becomes negative on cutting is an argument against the theory mentioned in a previous paper⁶ that injury causes an instantaneous exit of sap which might account for the results without the necessity of assuming the presence of layers in the protoplasm. The coming out of sap at B could not in any case do more than bring B to the same condition as C and so reduce the potential difference between them to zero; it could not make B negative as is the case here (since sap in these experiments acts like 0.05 M KCl).

A great variety of combinations were tried which need not be described in detail since the results were in every case in accord with the theory previously developed. We may conclude that the theory serves a useful purpose in bringing all the facts under a single viewpoint and in enabling us to predict the behavior of the cell under a great variety of conditions.

SUMMARY.

The hypothesis of protoplasmic layers enables us to predict the bioelectrical behavior of the cell under a great variety of conditions. It is shown in the present paper that this is clearly the case when a death wave passes through different points in contact with unlike solutions.

⁶ Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 673.

THE VISCOSITY OF BLOOD SERUM, AS A FUNCTION OF TEMPERATURE.

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(Accepted for publication, June 16, 1928.)

The viscosity of colloidal solutions is not easily determined by the methods which involve the use of a glass capillary. The technical difficulties encountered are important, and when they are successfully surmounted they still require a relatively large amount of liquid. Moreover, they do not permit the continuous observation of the variations which may take place in a solution as a consequence of a reaction, or under the influence of temperature, or of time. These were the main reasons which led us to establish a viscometer which would escape the above limitations. Such an instrument was described a few years ago.¹ Suffice it to say that it is based on the principle of two coaxial cylinders; the outside cylinder or cup, which contains about 1 cc. of the solution to be studied, is rotating at constant speed, and the inside cylinder, or bob, is suspended by means of a galvanometer wire. A mirror, supported by the suspension, a lamp and scale for the readings and a good thermostatic arrangement, are provided.

The purpose of the present paper is to report the results of roughly 167 series of experiments, representing about 17,000 readings of viscosity, performed with normal blood serum (rabbit, dog, horse), and to discuss the interpretation of some of the data obtained, with especial reference to the hydration of the serum proteins.

EXPERIMENTAL.

In order to save the reader's time, and to make him visualize a series of experiments at a glance, only charts will be published. The experiments chosen for publication are representative of the others, as no radical departure from the mean has been observed so far.

¹ du Noüy, P. L., *J. Gen. Physiol.*, 1923, v, 429.

The experiments were carried on in the following way: the serum was poured into the cup (1 cc.) and the bob lowered into the serum. The zero being checked, the motor was started, and a first reading made at the starting temperature (between 20° and 25°C.). After checking the zero again, and repeating this measurement at least 3 times, with an interval of 5 minutes between each reading, the heat was put on. (A current of 1 ampere through a resistance immersed in the oil surrounding the cup brought the temperature up from 20° to 70° in about 45 minutes.) Then the readings were taken, and recorded simultaneously with the temperature. A telescope placed alongside of the scale makes this possible. As a rule, unless some critical point was neared or some unforeseen phenomenon occurred, readings were taken every 2°. The current was left on until the temperature of 70°C. was reached, in the first series of experiments (Figs. 1 and 2); in all other experiments reported in this paper the heat was stopped after a certain temperature had been reached (50°, 55°, 56° to 60°) and the oil allowed to cool by itself. As a rule, in order that the cooling from, say, 55° to 20°, should take about the same time as the heating from 20° to 55°, cold water was made to circulate in the double wall of the oil bath, and the rate controlled accordingly.

The first set of experiments is shown in Fig. 1. The ordinates express the readings on the scale, and therefore are arbitrary figures, but proportional to the absolute viscosities. In order to give an idea of the order of magnitude, the curve expressing the viscosity of water as a function of temperature is drawn below.

A simple glance at this chart immediately reveals the presence of a critical point, corresponding to an absolute minimum of viscosity, at a temperature near 56°. Had we not known beforehand that this was a critical temperature for the serum, from a biological standpoint, we could not have failed to notice it from these curves.

Fig. 2 illustrates the same phenomenon. The turning point may vary, from 56° to 58°. But in the great majority of cases, the viscosity reaches its minimum value between 56° and 57°, stays constant up to 58°, sometimes up to 59°, and begins to increase more rapidly than it decreased before. From 62° to 65° the increase becomes very rapid, and it sometimes happens that the spot has left the scale before 70° is reached. This is usually the case with horse serum (which is normally more viscous than rabbit serum). It happens rarely in the case of rabbit serum. Fig. 2 shows that it was necessary to keep the temperature at 70° for 2 minutes in order to send the spot off the scale. It is to be noted that when fresh serum is used, important fluctuations

are frequently observed around 45° to 55° . These were *never* observed when the serum had been submitted to a heat of 55° , even for 5

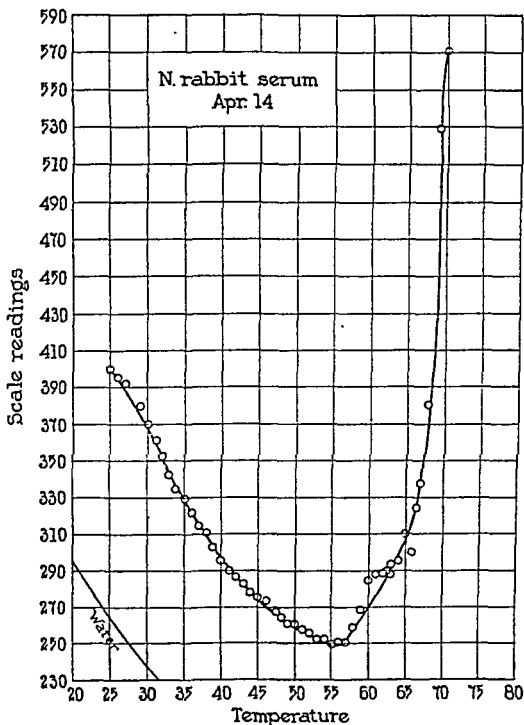


FIG. 1.

minutes, or when it was old. It appeared to us that it might be interesting to follow the phenomenon more closely, and to try and de-

termine whether the temperature played a specific part in the changes of viscosity, or whether the time of heating was the capital factor. In other words whether heating for 15 minutes at 55° would produce the same increase in viscosity as 5 minutes at 60° , for example. The first experiment was made with a serum heated at 55° for 10 minutes in a sealed tube. It is obvious from Fig. 2 that there is no fundamental change in the curve: the two control curves (white and black circles) almost coincide with that of the heated serum.

Another similar experiment is shown in Fig. 3. However instead of carrying the heating on up to the time when the spot goes off the scale, it was stopped, and the liquid was allowed to cool, according to the technique previously described. The white circles express the values of the heated serum 1 hour at 50° . The black circles are the values taken by the heated serum, on cooling, after having been kept at 57° for 5 more minutes. In general, no difference can be detected between heated and unheated serum when the serum is not heated above 55° . Up to 50° the curve expressing the viscosity of serum as a function of temperature is parallel to that of pure water, and its proteins play no part at all, or rather act only by their bulk, to displace the curve as a whole. From 50° on, a slight departure is observed; it goes on increasing until the minimum value is attained around 56° . Heating for 1 hour at 50° fails to alter permanently the viscosity of serum. The phenomenon is entirely reversible, as in the preceding case (Fig. 3).

- Heating for 15 minutes at 55° acts in the same way. But as the heat was brought up to 56° in one series of measurements, while it was stopped at 55° in the other (Fig. 4) a small but marked difference could be detected between the two cooling curves. Taking evaporation into consideration, the slight increase in viscosity of the serum heated up to 55° can be accounted for, but the difference between this sample and the sample brought up to 56° is due to something else. Here the phenomenon is no longer reversible; the relative viscosity reaches 1.70. The same experiment was repeated with the same serum, heated for 15 minutes at 56° in a sealed tube. The mean viscosity was 1.70. The increase is small, but constant with this serum. It was not always observed with other sera. We can therefore state that, from our experiments, it appears that 56° is the lowest

temperature at which an irreversible phenomenon affecting its viscosity occurs in rabbit serum, in 15 minutes. However, an exception to this rule was found once. But if the heat is kept for 30 minutes at 56° (Fig. 5) the mean value of η climbs up to 1.77 and higher still on cooling. On the other hand in certain cases, 5 minutes at 56° (dog serum) may bring forth no change at all.

Another serum, the viscosity of which was normally high, heated up to 58° and cooled immediately after that temperature was reached, showed no modification. Serum 3 ($\eta = 1.63$) heated at 58° for 15 minutes, gave similar curves with $\eta = 1.80$. Half an hour at 58° brings the value of η up to 1.95.

Another serum heated for 1 hour, and 2 hours, at 58° (Fig. 6) reaches a viscosity of 1.85 (mean value) with a maximum of 1.90. 1 hour's heating of this serum at 60° fails to affect it more than 1 hour at 58°.

Fig. 7 is self-explanatory. The serum was heated in the cup, for 5 minutes, in all cases. As the temperature goes up, the different samples show a similar behavior. But on cooling, the differences between the different curves, according to the temperature reached, go on increasing. However, up to 62° no sign of structure in the liquid was found. The zero checked perfectly, and what was measured was true viscosity.

Fig. 8 summarizes for one animal a whole series of observations, showing which combinations of temperature and of time determined the same increase in viscosity. The question then arises as to what the increase in viscosity is due to, and how it can be interpreted.

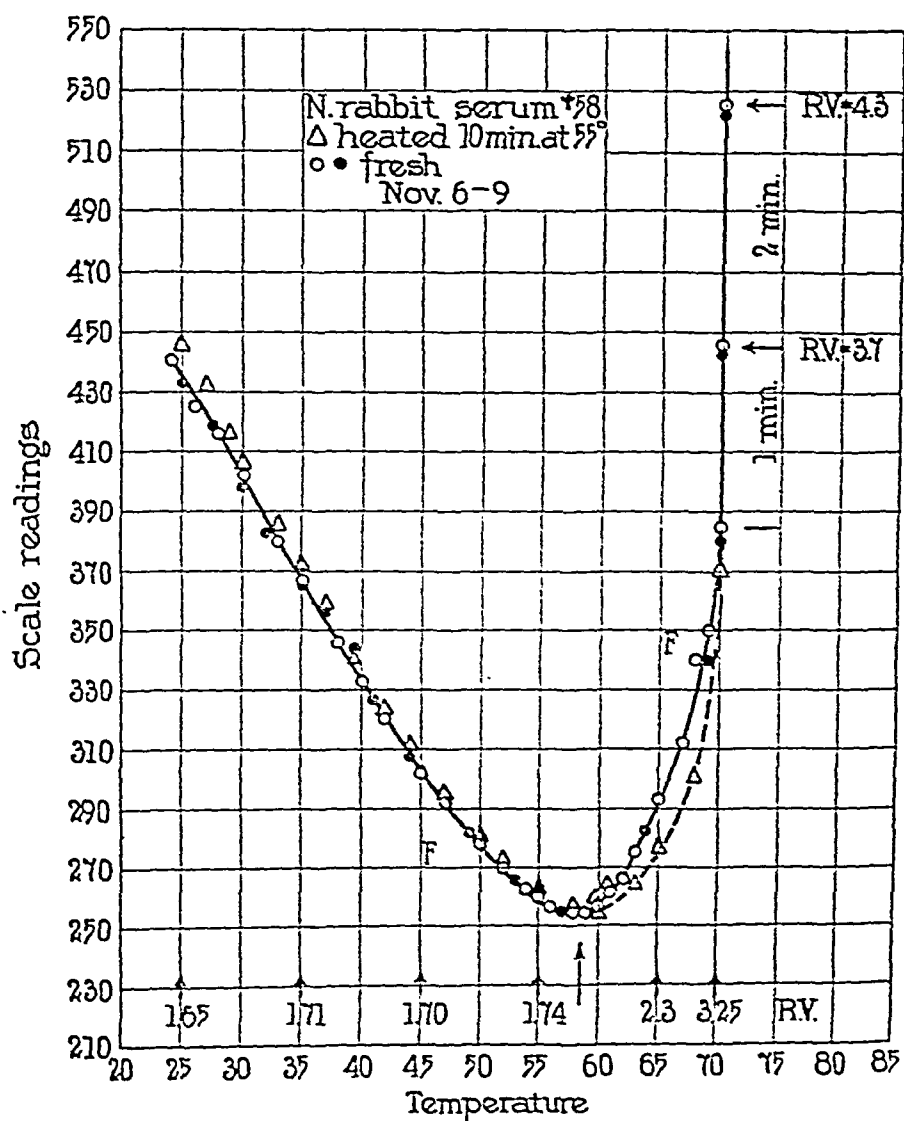


FIG. 2.

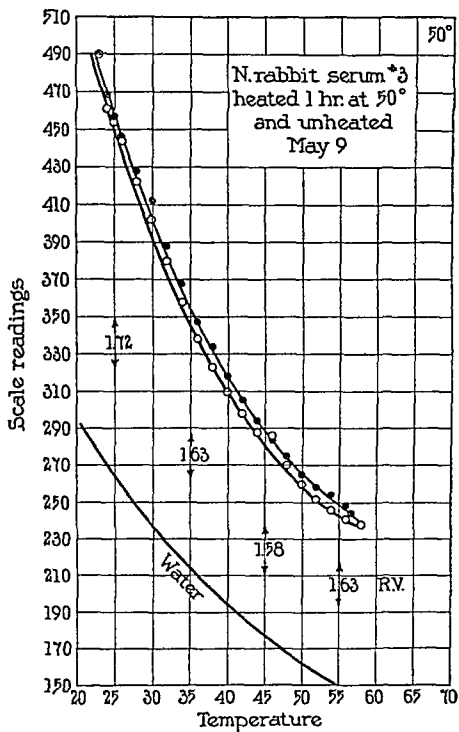


FIG. 3.

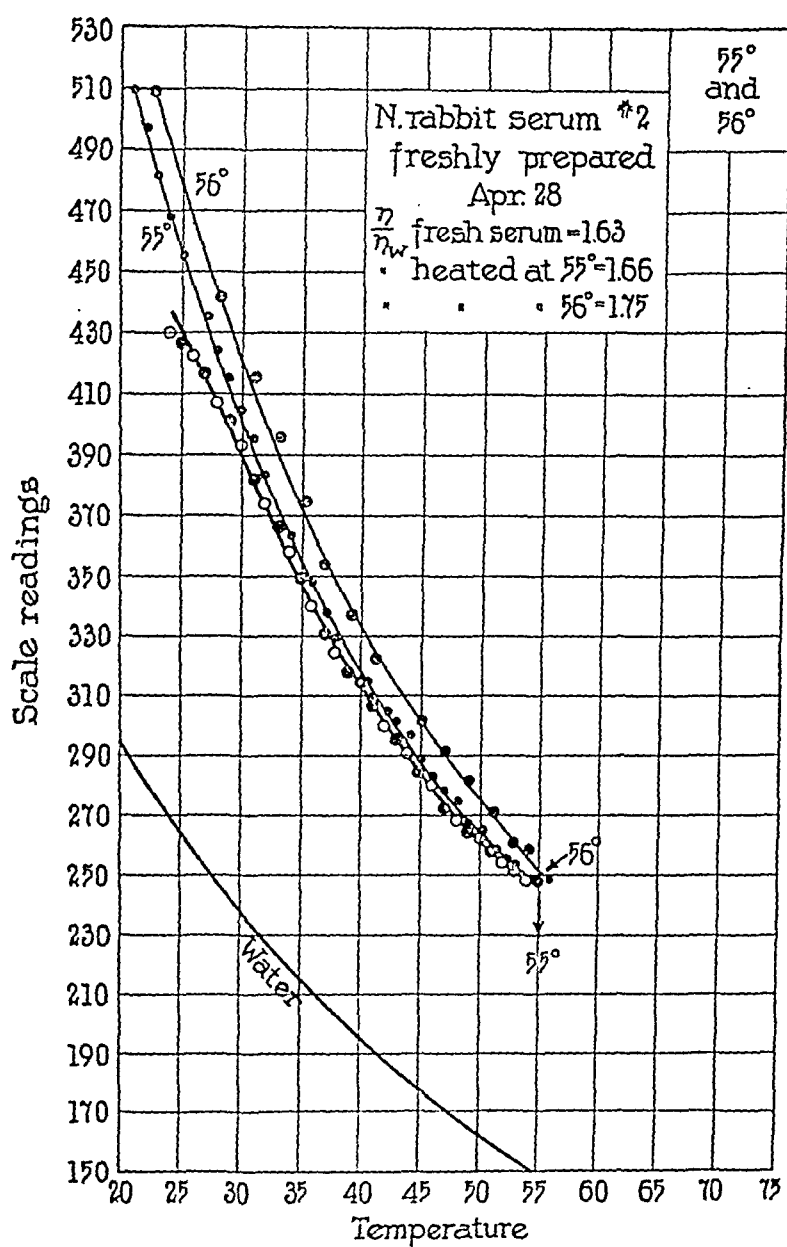


FIG. 4.

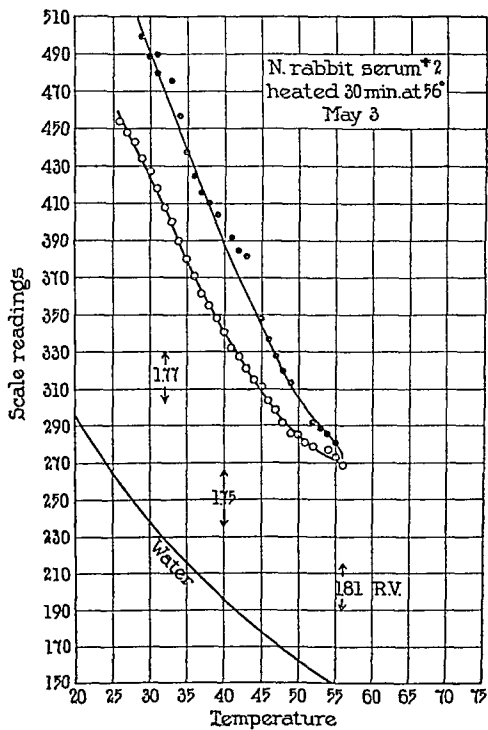


FIG. 5.

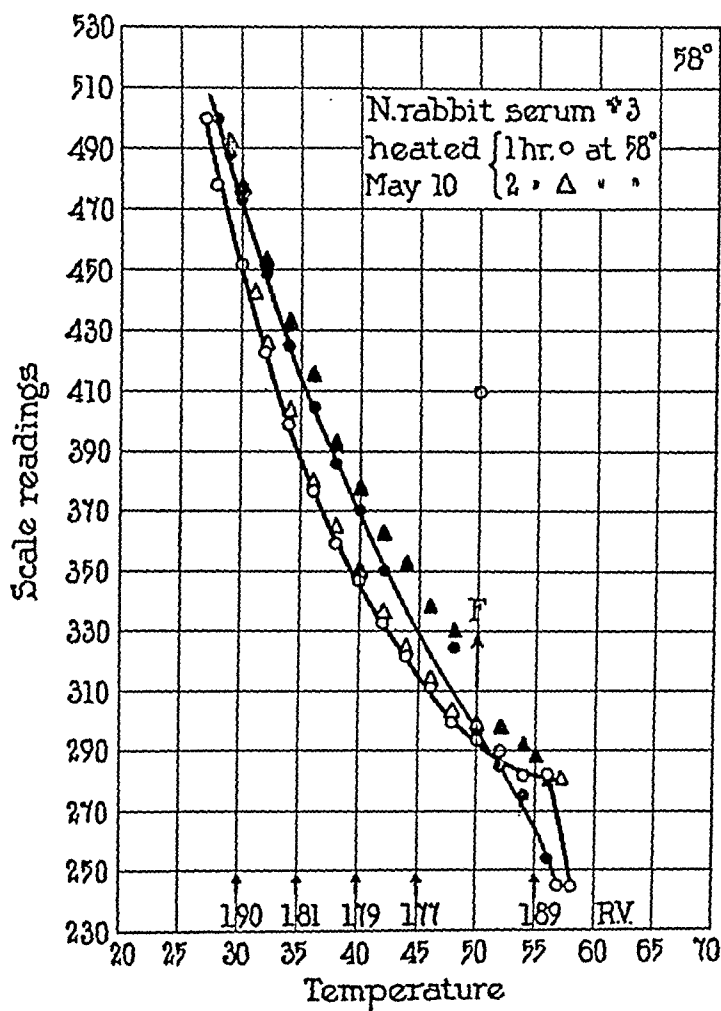


FIG. 6.

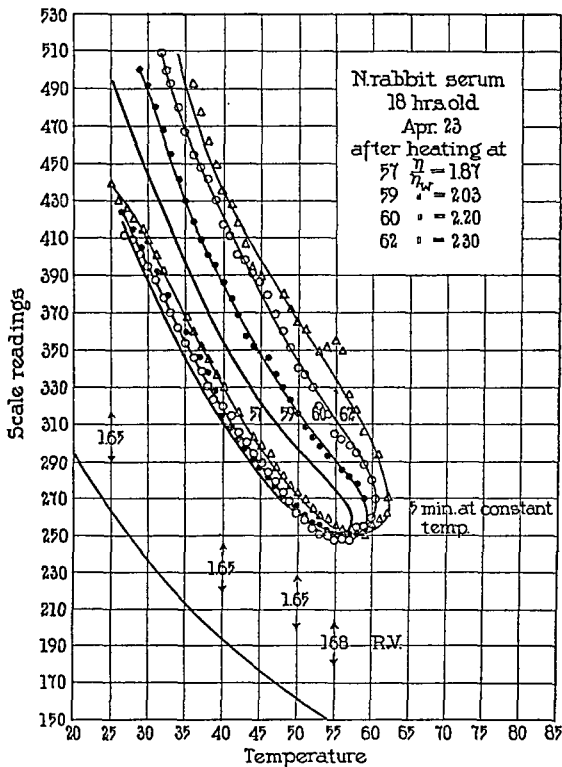


FIG. 7.

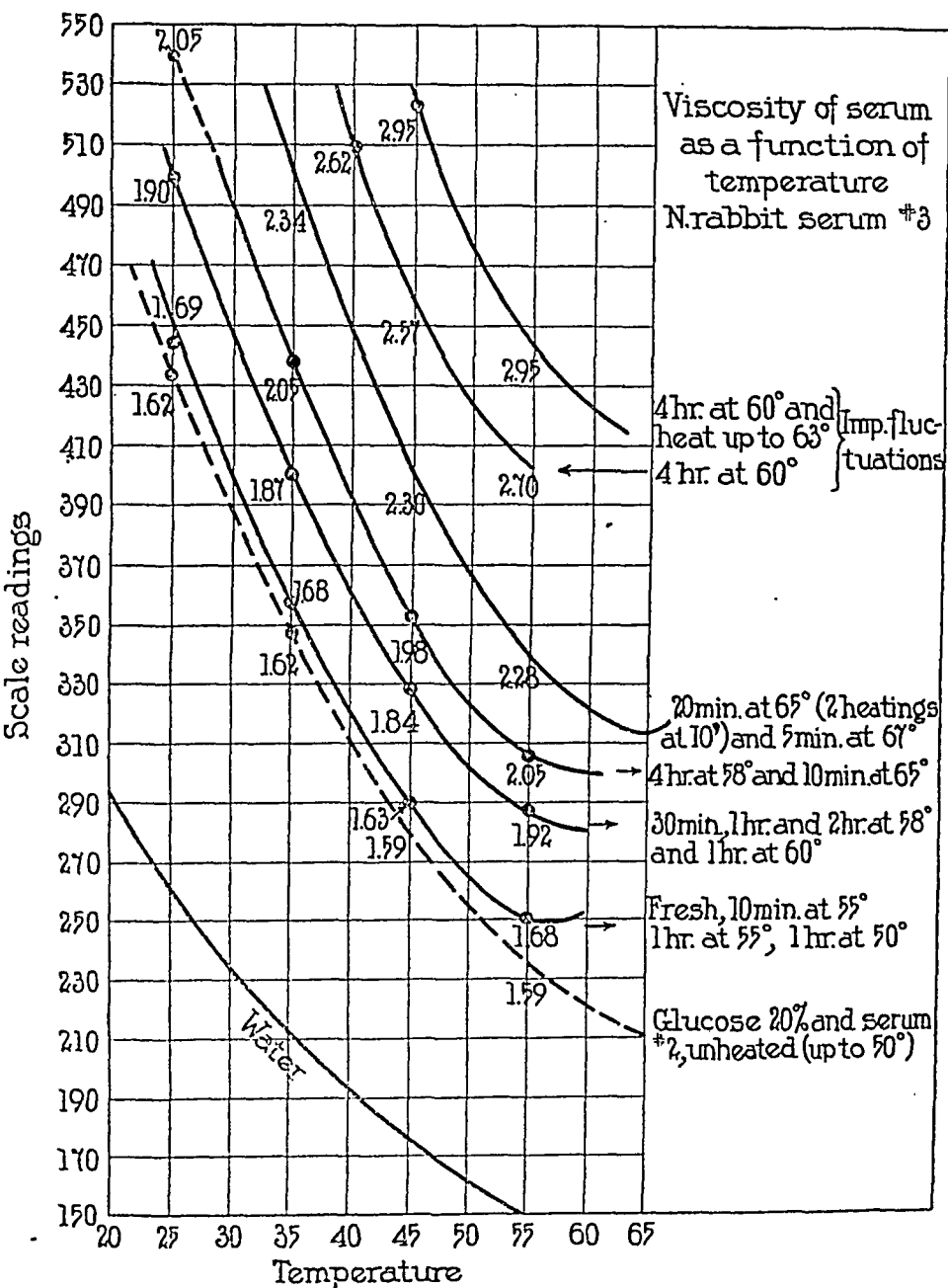


FIG. 8.

DISCUSSION.

In our experiments the concentration remains constant, yet the viscosity increases. On the other hand, Einstein² states that the degree of dispersion, *i.e.*, the size of the molecules or particles is immaterial, and that viscosity can be expressed as a linear function of the volume fraction of the dispersed substance, according to the formula

$$\eta = 1 + 2,5 \varphi \quad (1)$$

which is an approximation of the expression

$$\eta = \frac{1 + 0.5 \varphi}{(1 - \varphi)^2} \quad (2)$$

where η stands for the relative viscosity of the suspension, and φ for the volume occupied by the dispersed substance expressed as a fraction of the total volume of the solution. These equations, however, fail to express the experimental facts. The reason for this lack of agreement is not clear. But Kunitz has recently proposed an empirical formula which applies remarkably well to a number of widely different cases, including lyophilic and lyophobic sols, and within large limits of concentration. This formula is³

$$\eta = \frac{1 + 0.5 \varphi}{(1 - \varphi)^4} \quad (3)$$

The problem we are facing can be stated as follows: Assuming that Einstein's view concerning the part played by φ as defined above is correct, and that the degree of dispersion is immaterial, we can use formula (1), and compute the values of φ , and consequently determine $\frac{\varphi}{C}$ which expresses the specific volume of the solute (proteins). These values divided by the specific volume of the dry proteins should give the amount of hydration of the proteins in solutions, at temperatures up to 55°, and its increase as a function of temperature. But we know that this formula does not apply to lyophilic sols at high concentration, consequently, we cannot rely on the figures at all.

² Einstein, A., *Ann. Physik.*, 1906, xix, 289; 1911, xxxiv, 591.

³ Kunitz, M., *J. Gen. Physiol.*, 1926, ix, 715.

On the other hand we can apply Kunitz's formula (Table I) which we know fits the experimental facts very satisfactorily but then the main assumption of Einstein concerning the rôle of the degree of dispersion, which is a consequence of his mathematical derivations, may not hold any longer. Therefore, it does not seem possible, at present, to decide whether the figures computed in this way express quantitatively the increase due to hydration alone or whether some other

TABLE I.

Viscosity of Rabbit Serum 3, Heated at Different Temperatures, and Values of φ and $\frac{\varphi}{C}$ Computed from Kunitz's formula (see Figs. 7 and 8).

C = mean concentration of proteins in the serum = 6.5 per cent.

1	2	3	4	5	6	7
Relative viscosity $\frac{\eta}{\eta_0}$	φ	Specific volume $\frac{\varphi}{c}$	Specific volume of dry proteins	Ratio Column 3 Column 4	Increase per cent of specific volume (Hydration ?)	Increase in hydration due to heating
	<i>per cent</i>				<i>per cent</i>	<i>per cent</i>
1.65	10.7	1.645	0.785	2.09	109	Unheated
1.69	11.2	1.720	"	2.19	119	
1.88	13.3	2.030	"	2.58	158	
2.03	14.8	2.275	"	2.90	190	
2.30	17.2	2.650	"	3.37	237	
2.62	19.8	3.050	"	3.88	288	
2.95	21.8	3.350	"	4.27	327	

phenomenon is also responsible for it to a certain extent. However, column 6 has been tentatively designed as "hydration."⁴

⁴ The influence of the charge of colloidal particles on the viscosity of the sol has been taken in consideration, especially by von Smoluchowski (*Kolloid-Z.*, 1916, xviii, 194.) who enlarged Einstein's formula which became:

$$\eta = \eta_M \left\{ 1 + 2.5 \varphi \left[1 + \frac{1}{\lambda \eta_M r^2} \left(\frac{D \zeta}{2 \pi} \right) \right] \right\}$$

however our results are not in accord with this formula, which should lead to a lower viscosity as the size of the particles increases. We observe the contrary, and there seems to be no doubt that after heating the size of the particles increases, since coagulation is the limit of the phenomenon. Perusal of Freundlich's excellent discussions of the subject in general (*Colloid and capillary chemistry*, New York, pp. 367 and following; 539, etc.) will repay the reader.

All that can be said is that it expresses the increase per cent of the specific volume of the serum proteins. It is interesting to note that the figures expressing "hydration" obtained from Einstein's formula (3) are larger (3.71 times) than those obtained from Kunitz's formula (1), and that this ratio remains constant up to a viscosity of 2.03. From this figure up the discrepancy begins and increases rapidly with increasing viscosity.

Fig. 8 suggests another observation: the dotted curve (viscosity 1.62) was obtained with a sugar solution (glucose 20 per cent). The concentration is 3 times that of the serum. Hence, serum may be said to behave exactly like a true solution, as a function of temperature, up to nearly 55°. It is surprising that such a concentrated solution of proteins should have such a low viscosity. When, by diluting the serum, its specific volume is made equal to that of the sugar solution, its viscosity is much inferior to that of the latter.

FRACTIONATION OF GELATIN.

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(Accepted for publication, July 2, 1928.)

It has been shown by the writers¹ that such phenomena as the swelling of electrolyte-free gelatin blocks, the high viscosity of gelatin solutions, the decrease of the pH effect on viscosity with increase in the concentration of gelatin solutions, and, finally, the peculiar phenomenon of shrinking of dilute gels when placed in cold water, are explainable on the assumption that gelatin consists of a mixture of at least two substances, one of which is soluble in cold water, while the other is insoluble even in warm water. Actually, it has been found possible to isolate from gelatin by means of alcohol precipitation two fractions which differ enormously in their physical properties.² One of the fractions has properties similar to those of albumin; it is soluble even in ice water, does not set to a gel, has a low viscosity, and does not give rise to the pH-viscosity curve characteristic for gelatin. Besides, it has an osmotic pressure at its isoelectric point (pH 4.7) considerably higher than that of isoelectric gelatin, from which it has been isolated. The second fraction has properties opposite to those of the first one; it is insoluble in cold water, sets to a gel at a low concentration, swells much less, and has a higher viscosity and lower osmotic pressure than ordinary gelatin. But this fraction is still "soluble" in warm water.

It had been previously found by Schryver³ and his associates that a soluble material could be obtained from gelatin by allowing isoelectric salt-free gelatin to stand at 14–16°C. Under these conditions the

¹ (a) Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 161. (b) Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 811. (c) Northrop, J. H., *J. Gen. Physiol.*, 1926–27, x, 893. (d) Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1926–27, x, 905. (e) Kunitz, M., *J. Gen. Physiol.*, 1928–29, xii, 289.

² Northrop and Kunitz,^{1a} p. 167.

³ Knaggs, J., Manning, A. B., and Schryver, S. B., *Biochem. J.*, 1923, xvii, 473.

gelatin separates out as a clot leaving some soluble protein material in the supernatant fluid.⁴ If the precipitate is then suspended in water a lower concentration of the soluble material is found in the supernatant solution. This solution moreover did not have the properties of ordinary gelatin, so that, as these workers point out, it can not be considered as a saturated solution of gelatin. These experiments were repeated and confirmed by the present writers, but it was found that if the gelatin were allowed to stand at 23° instead of 15° a very much larger amount of the soluble material was obtained and this fact was used as the basis of the method of separation used in the present paper.

Experimental Procedure.

A stock of isoelectric gelatin is prepared from Cooper's non-bleached gelatin by the modified Loeb's method as described elsewhere.⁶ A solution of 60 gm. air-dried isoelectric gelatin (85 per cent dry weight) is made in 3000 cc. of distilled water, which has been adjusted to pH 4.7 by means of acetic acid, filtered through cotton wool into Pyrex flasks, and put into a water bath kept at a constant temperature of 23°C. A layer of toluene is added to the gelatin to prevent mould and bacterial growth. The gelatin solution gradually turns milky white, thickens, and after several days the thick white gel begins to break up into a gelatinous precipitate which settles slowly. The precipitation may be accelerated by stirring up the gel occasionally. After 5 or 6 days the flasks are removed from the bath, the supernatant solution is decanted as much as possible, and the rest is centrifuged, care being taken not to heat the gelatin in the centrifuge. The centrifuged precipitate is stirred up once or twice in the centrifuge bottles with cold water of pH 4.7 and recentrifuged. It is then melted and made up to about 80 per cent of the original volume of the 2 per cent solution with water of pH 4.7, and allowed to set at 23°C. for the second precipitation. The process is repeated 12 to 15 times until a precipitate is left which does not dissolve even at 50°C. Care has to be taken during the whole process that the pH should remain at 4.7. The final precipitate is washed several times in the centrifuge bottles with hot water, and transferred into 95 per cent alcohol, where it is allowed to remain overnight. The alcohol is then decanted off and the gelatin is ground up in a mortar with ether, which is allowed finally to volatilize.

⁴ Schryver and Thimann⁵ were also able to obtain a soluble and insoluble fraction by means of electrolysis of gelatin solutions.

⁵ Schryver, S. B., and Thimann, K. V., *Biochem. J.*, 1927, xxi, 1284.

⁶ Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1927-28, xi, 477. Gelatin prepared in this way is practically free from diffusible nitrogenous material, cf. Knaggs, Manning, and Schryver.³

The air-dried precipitate is a fine powder of a light chocolate appearance. The yield is about 1 per cent of the original amount of gelatin.

TABLE I.
Precipitation of Isoelectric Gelatin at 23°C.

	Volume	Dry gelatin	Strength of solution
	cc.	gm.	per cent
Original.....	3000	51	1.66
1st precipitate.....	2100	43.5	2.07
1st supernatant solution.....	900	6.9	0.77

TABLE II.
Influence of Initial Concentration of Gelatin Solution on Concentration of Gelatin in the Supernatant Liquid after 1st Precipitation at 23°C.

Initial concentration in gm. dry gelatin per 100 gm. solution.....	0.6	1.17	1.70	2.35	2.93
Gm. dry gelatin per 100 gm. supernatant liquid	0.19	0.53	0.68	1.21	1.19

TABLE III.
Gelatin Found in Supernatant Liquid on Repeated Precipitation of a Gelatin Solution at 23°C. Original Amount of Gelatin 51 Gm. by Dry Weight.

Precipitation	1	2	3	4	5	6	7	8
Concentration of gelatin in supernatant liquid in gm. per 100 cc. solution.....	0.77	0.55	0.48	0.50	0.50	0.40	0.37	0.42
Gm. gelatin in the supernatant liquid..	6.9	9.5	5.6	1.2	3.1	4.7	2.3	1.9
Precipitation.....	9	10	11	12	13	14	15	
Concentration of gelatin in supernatant liquid in gm. per 100 cc. solution.....	0.31	0.31	0.24	0.15	0.15	0.15	0.12	
Gm. gelatin in the supernatant liquid ..	1.9	2.0	1.5	0.9	0.98	0.61	0.54	

tin. The supernatant solutions may be used for preparation of the soluble fraction by means of alcohol, as described elsewhere.² If the precipitation is carried out at a lower temperature than 20°C. practically

all of the gelatin comes out as a precipitate with only a trace remaining in the supernatant solution. On the other hand, when the precipitation is done at 23°C. 14 to 18 per cent of the original gelatin remains in the supernatant solution. The distribution of gelatin between the precipitate and the supernatant solution after the first precipitation at 23°C. is shown in Table I.

The concentration of soluble material increases with the total concentration of gelatin, as shown in Table II.

On further precipitation the amount of gelatin in the supernatant solution gradually decreases, as shown in Table III.

The Physical Properties of the Separated Fractions of Gelatin.

Solubility.—The difference in the solubility of the various fractions of gelatin appears even after the first precipitation. Thus, in one of the experiments a solution of the first precipitate and of the first supernatant solution was completely precipitated by means of alcohol, washed with ether, and dried in the air. Suspensions were then made of 0.1 gm. dry powder of the two fractions in 10 cc. of water each, at 25°C. The fraction from the supernatant solution dissolved immediately in the water producing a clear solution, while the fraction from the first precipitate swelled but did not dissolve unless it was heated. After repeated precipitation the solubility of the precipitate becomes less until finally it does not dissolve even in hot water. The insoluble fraction appears only on repeated fractionation. Simple reheating of gelatin without separation of the precipitate does not produce any visible effect on the gelatin. A 2 per cent solution remained unchanged after heating to 50° and cooling to 23° more than 20 times during a period of 2 months.

Swelling of Powdered Gelatin.

The final precipitate does not swell when suspended in cold water. Its bulk is the same whether it is suspended in water, alcohol, or toluene. This is shown in Table IV. The bulk was determined by centrifuging suspensions of 0.1 gm. of the dry powder in 10 cc. of various liquids to constant volume of sediment. As a comparison suspensions were also made of ordinary isoelectric gelatin which had been precipi-

tated and dried by means of alcohol and ether in the same way as the fractionated gelatin, and also of the precipitate from the first precipitation.

Thus it is seen that when 0.1 gm. of ordinary gelatin is suspended in water it has a bulk of 0.9 cc. when centrifuged as compared with the bulk of 0.25–0.30 cc. when it is suspended in absolute alcohol or toluene; on the other hand, the insoluble fraction of gelatin from the final precipitation has practically the same bulk, 0.35–0.40 cc., no matter whether water, alcohol, or toluene is used. It is to be noted that the insoluble gelatin which had been precipitated only once gives higher swelling in water than ordinary gelatin, which is partly soluble at 25°C.

TABLE IV.
Comparison of Swelling of Various Types of Gelatin at 25°C.

Liquid	Insoluble gelatin from final precipitation	Insoluble gelatin from first precipitation	Normal isoelectric gelatin
	Cc. sediment		
Distilled water.....	0.40	1.20	0.90
Absolute alcohol.....	0.35	0.20	0.30
Toluene.....	0.35	0.25	0.25

Effect of Acid or Alkali on the Swelling of the Insoluble Fraction of Gelatin.

Addition of dilute HCl or NaOH to a suspension of the insoluble fraction of gelatin in water brings about a considerable swelling of the particles of gelatin, owing to the unequal distribution of the diffusible ions between the particles and the outside solution in accordance with the Donnan equilibrium. The swelling increases with increase in the amount of acid or alkali until a pH about 3.0 on the acid side or 9.0 on the alkaline is reached when the material begins to dissolve on heating, forming a viscous solution. On bringing the solution back to pH 4.7 the material rapidly precipitates out in a flocculent state.

Swelling of Gels Made of Mixtures of the Soluble and the Insoluble Fractions of Gelatin.

The mechanism of swelling of gels of isoelectric gelatin has been studied extensively by the writers.¹ The final conclusions reached may

be summarized as follows. There are three forces which affect the swelling of isoelectric gels:

1. The osmotic force due to the presence of a soluble ingredient of gelatin even at temperatures as low as 5°C. This force causes the block of gel to take up water, and is the significant factor in gels of high gelatin content.

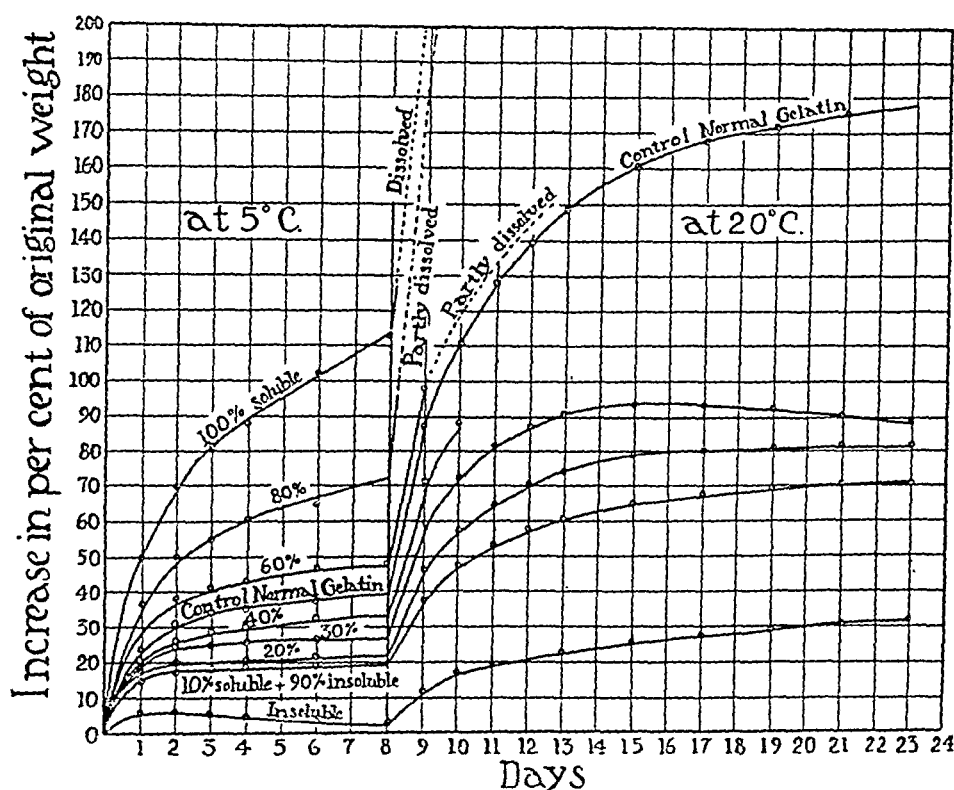


FIG. 1. Swelling of 30 per cent gels consisting of mixtures of insoluble and soluble fractions of gelatin. Blocks of gel weighing about 1.5 gm. each were allowed to swell in 200 cc. M/30 acetate buffer pH 4.7.

2. The residual elastic force in the micellæ, which originates while the gelatin is still in the sol state. This force causes the block of gel to lose water and shrink. It is the significant factor in dilute gels.

3. Finally the elastic force of the block of gel as a whole which resists both the swelling and the shrinking of gels.

According to this viewpoint a concentrated gel made up entirely of the insoluble ingredient should not swell at all in the absence of acid,

alkali, or salt, since there are no soluble molecules to produce an osmotic force. Addition of increasing amounts of the soluble fraction to a gel of the insoluble fraction of gelatin gives rise to osmotic pressures, and increased swelling should be expected. Fig. 1 shows what actually takes place when gels containing various mixtures of the insoluble and soluble ingredients were placed in a M/30 acetate buffer pH 4.7. The experiment was done as follows:

A series of solutions containing 30 gm./100 gm. of solvent was made up of various proportions of soluble and insoluble fractions in warm 0.075 N NaOH. 2 cc. of each solution was poured into a mould of glass tubing, mounted on a paraffin block, and allowed to set for 24 hrs. in the refrigerator at 5°C. The blocks of gel were then removed from the glass tubing, weighed, and put into 200 cc. of M/30 acetate buffer pH 4.7 which had been previously cooled to 5°C. The blocks were weighed at various intervals of time in a refrigerating room which was kept at constant temperature of 5°C. A block of 30 per cent ordinary isoelectric gelatin dissolved in 0.075 N NaOH was prepared and put into 200 cc. of M/30 acetate buffer pH 4.7 as a control. The blocks remained at 5°C. for 8 days, and then were transferred with the outside solutions to a constant temperature water bath of 20°C.

The plotted curves for the rate of swelling of the gels show that at 5°C. the gel consisting of 100 per cent insoluble fraction of gelatin swells slightly if at all, while the addition of the soluble ingredient brings about swelling. The swelling increases rapidly as the proportion of the soluble material increases. Gels consisting of more than equal parts of soluble and insoluble fractions swell more than the normal gelatin control. When transferred to 20°C. all gels begin to swell faster, and those that have been swelling more than the control at 5°C. swell at 20°C. so rapidly that in a day or so they dissolve completely leaving a residue of insoluble gelatin. The gel consisting entirely of the soluble fraction melts immediately when the temperature is raised to 20°C. That the fractionation is not complete yet is shown by the fact that even the 100 per cent *insoluble* gel swells somewhat at 20°C. which indicates that there is still left in it some of the fractions which are soluble at 20°C. It is also possible that the presence of the electrolytes of the buffer affect the solubility of the gelatin as was found before in the case of the swelling of ordinary gelatin. The fractionation of the soluble fraction used in this experiment was purposely stopped at this

point so that a 30 per cent gel at 5°C. would be obtained. By means of further fractionation of the soluble ingredient with alcohol it is very easy to obtain a gelatin fraction which does not set even at 5°C. no matter how concentrated the gel is.

Negative Swelling of Dilute Gels of Insoluble Gelatin.

When gels containing less than 10 per cent gelatin are placed in cold water or dilute buffer pH 4.7 the gels not only do not swell but actually shrink and lose water. It has been shown by one of the writers¹⁶ that the force causing the dilute gels to lose water is the elasticity in the micellæ of gelatin. This elastic force, which is brought about by the hydration of the micellæ in the gelatin solution, is due, first, to the presence of soluble gelatin inside of the micellæ and, second, to an excess of diffusible ions inside of the micellæ over the outside solution. The osmotic pressure in the micellæ is therefore higher than in the surrounding solution and water enters until the elastic force is equal to the osmotic pressure. When the gelatin solution sets to a gel the soluble material precipitates and the ions diffuse out when the gel is placed in water. The osmotic pressure is thereby destroyed and the water is forced out by the elasticity of the micellæ, thus bringing about a gradual shrinking and loss of water by the block of gel as a whole. This contraction is greater the greater the amount of water originally held by the micellæ. The amount of water held in them is large in gelatin sols made up in acid or alkali. In ordinary gelatin the micellæ form only a part of the total gelatin, the rest of which is found in solution outside of the micellæ. A solution of the *insoluble* fraction of gelatin in dilute NaOH is not a perfect solution but rather a colloidal suspension of swollen micellæ, as shown by the high viscosity of the mixture. The number of micellæ per gm. of substance then is greater in the case of the insoluble fraction of gelatin than in the case of ordinary gelatin. Hence it is to be expected that when dilute gels are made up of both materials the one consisting of the insoluble component of gelatin should lose more water when put in dilute buffer pH 4.7 than the gel which was made up from ordinary gelatin. That this is actually what happens is shown by the following experiment.

Two solutions were made up each containing 0.1 gm. gelatin + 2 cc.

M/10 NaOH. One was made up of ordinary isoelectric gelatin, while in the other the insoluble fraction was used. Both solutions were allowed to set for 24 hours at 5°C. and then the gels were weighed and each put into 250 cc. M/30 acetate buffer pH 4.7 at 5°C. where they remained for about a month. The weight of the gels was determined at various intervals of time. Fig. 2 shows the results. It is seen that while the block of gel which was made up of ordinary gelatin has lost finally about 22 per cent of its original weight, the one that was made up of the insoluble fraction has lost as much as 68 per cent under the same conditions. The dry weight of the gel was unchanged so that the result is not simply due to solution.

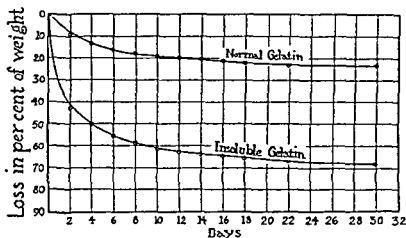


FIG. 2. Negative swelling of 5 per cent gels in M/30 acetate buffer pH 4.7 at 5°C.

The Irreversibility of Gelatin Fractionation.

According to the mechanism of swelling outlined above the soluble ingredient of ordinary gelatin is present both inside and outside of the micellæ. Each micella may be compared to a cell, filled with soluble gelatin; the cell wall, which is impermeable to the soluble gelatin, consists of the insoluble fraction of gelatin. The number of micellæ in a gelatin solution increases with the decrease in temperature until the setting point is reached. When a dilute solution of salt-free isoelectric gelatin is left at a temperature of about 23°C. the micellæ together with the contained soluble fraction of gelatin in them gradually settle down as a gelatinous precipitate, while the supernatant liquid consists of a

solution of practically pure soluble material. The process of removal of the soluble fraction of gelatin from inside of the micellæ is a difficult one owing to the impermeability of the micella wall. Only through a long series of reprecipitations accompanied by repeated heating of the solution is it possible to obtain an insoluble fraction which is apparently free from the soluble component. The liberation of the soluble material is probably due to a rupture of the micellæ by osmotic pressure. In a gelatin solution some soluble material exists both inside and outside of the micellæ. When the gelatin is allowed to set in dilute solution some of the soluble material escapes and is removed. On remelting the gelatin the osmotic pressure difference between the inside and outside of the micellæ is greater than before and they therefore swell more. That this actually happens is shown by the fact already mentioned that a partially purified fraction has a higher viscosity than the original gelatin. As the purification proceeds the swelling increases until the micellæ rupture and liberate the soluble material. The ruptured micellæ thus give rise to the insoluble fraction of gelatin. This fraction is not only insoluble in hot or cold water in the absence of acid or alkali, but it will not mix even with the soluble fraction or with ordinary gelatin to form a homogeneous solution unless it is first dissolved in acid or alkali; on bringing the solution back to pH 4.7, however, the insoluble fraction precipitates out again. In order to resynthesize gelatin from its final fractions it is not enough to mix the fractions in the right proportions but it is necessary to reintroduce the molecules of the soluble material into the insoluble particles; or, in other words, to fill again the micellæ cells with a substance for which the cell wall is impermeable. This may be partially accomplished in the case of a solid block since the soluble material is held in the network of micellæ forming the block but it has not been possible to introduce the soluble material into the individual micellæ. It is probable that in the manufacture of gelatin the soluble material is actually formed inside the micellæ.

It could be assumed that the soluble fraction was formed from the gelatin by an irreversible chemical reaction and hence mixing the fractions would not give the original gelatin. This assumption appears improbable since, as was shown, repeated heating and cooling does not result in the separation of the two fractions. It has also been found by

Alsberg and Griffing⁷ that gelatin becomes partly soluble in cold water after prolonged grinding. If the grinding were sufficient to rupture the micellæ, the soluble material would be liberated.

Isolation of an Insoluble Substance from Gelatin by Partial Acid Hydrolysis.

A substance similar in many respects to the insoluble ingredient of gelatin described above is obtained when a 5 per cent solution of gelatin is partially hydrolyzed in M/10 HCl at 90°C. for about 2 hours. At this stage the gelatin no longer sets when cooled. On neutralization of the solution a flocculent precipitate appears, which can be centrifuged off and washed with water. On drying with alcohol and ether it gives a light brown powder similar to the one obtained by fractional precipitation. The yield is about 0.2 per cent. The powder is insoluble in hot or cold water, and swells considerably on addition of acid or alkali to its suspension in water until it is finally dissolved, as is the case with the insoluble component of gelatin from fractional precipitation. The only difference between the two substances is their behavior with regard to setting. It has been mentioned before that when an alkaline solution containing 5 per cent or more of the insoluble fraction of gelatin is left to cool to 5°C. it gradually sets to a gel. This does not happen with the insoluble substance obtained by acid hydrolysis of gelatin. A solution of it in dilute NaOH is very viscous, but it does not set even at a concentration of 10 gm. per 100 cc. solution. It is possible that the setting of gelatin depends on the presence of an ingredient which still exists in the material obtained by fractional precipitation but which is destroyed on hydrolysis with acid.

SUMMARY.

1. It is possible to fractionate gelatin by means of reprecipitation at 23°C. of a salt-free solution of pH 4.7 into two fractions, one of which is soluble in water at any temperature, and a second one which does not dissolve in water even when heated to 80°C.
2. The proportion of the soluble fraction in gelatin is much greater than of the insoluble one.
3. The insoluble fraction of gelatin does not swell when mixed with water, but it does swell in the presence of acid and alkali which finally dissolve it.
4. Blocks of concentrated gel made by dissolving various mixtures of

⁷ Alsberg, C. L., and Griffing, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1925-26, **xxiii**, 142.

the soluble and insoluble fractions of gelatin in dilute NaOH swell differently when placed in large volumes of dilute buffer solution pH 4.7 at 5°C. The gel consisting of the insoluble material shows only a trace of swelling, while those containing a mixture of soluble and insoluble swell considerably. The swelling increases rapidly as the proportion of the soluble fraction increases.

5. A 5 per cent gel made up by dissolving the insoluble fraction of gelatin in dilute NaOH loses about 70 per cent of its weight when placed in dilute buffer pH 4.7 at 5°C. A similar gel made up of ordinary gelatin loses only about 20 per cent of its weight under the same conditions.

6. It was not found possible to resynthesize isoelectric gelatin from its components.

7. An insoluble substance similar in many respects to the one obtained by reprecipitation of gelatin is produced on partial hydrolysis of gelatin in dilute hydrochloric acid at 90°C.

DARK ADAPTATION AND THE LIGHT-GROWTH RESPONSE OF PHYCOMYCES.

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I.

It has long been known that certain plants respond to an increase in intensity of illumination by a brief increase or decrease in growth rate. The increase was first shown by Blaauw (1914) for the unicellular, light-sensitive sporangiophores of *Phycomyces*, and simultaneously the decrease by Vogt (1915) for the etiolated coleoptiles of *Avena*.

If a sporangiophore of *Phycomyces* growing vertically in the dark is illuminated from above, or laterally from a number of opposed sources of equal intensity, after a definite interval an increase in the vertical rate of growth is observed, later followed by a decrease and brief fluctuations until the original rate is regained (see Fig. 1). This response has been called by Blaauw the "light-growth" response. If the light is maintained, the sporangiophore which has regained its original growth rate is then insensitive to that particular light, or "light-adapted." In order to produce a new growth response the intensity of the light must be again increased by a definite amount. The inverse phenomenon of "dark-adaptation" is shown by an increase of sensitiveness to stimulation by light as the sporangiophore is kept in the dark.

In the absence of precise studies of the kinetics of photic excitation and adaptation in plants it is of interest to examine the course of dark adaptation in *Phycomyces*, particularly as in the sporangiophores of this form the whole mechanism of reception and reaction is contained within one cell wall, and is probably confined to a very small region of that cell. Thus Errera (1884) showed that the whole growing zone of

the sporangiophore of *Phycomyces* was within 1.0 mm. of the sporangium, and Blaauw (1918) that the light-sensitive region probably did not extend more than 2.0 mm. below it.

II.

Pure cultures of *Phycomyces blakesleanus* Burgeff ("+" strain) were grown in short glass vials as previously described (Castle, 1927-28). For experimentation a culture was placed in a moist glass cell with plane walls and cover nearly submerged in a water thermostat enclosed by a dark room. The temperature of the bath was held at 24.4°C. throughout, the atmosphere of the cell being saturated with water vapor at that temperature. By means of a sliding block either of two incandescent bulbs controlled by separate switches could be brought directly above the culture. One of low intensity was used as an initial adapting light, the other high intensity bulb being later pulled into position and used for brief stimulation. Heat was screened from the culture by an interposed plate of colloidal gold glass. The particular sporangiophore under consideration was observed laterally against an intermittent red light by means of a horizontal microscope which projected into the dark room enclosing the thermostat and contained an ocular micrometer scale against which elongations of as little as 10μ could be noted.

An actual experiment was run as follows: a culture placed in the observation cell was allowed to become thoroughly adapted thermally and photically for at least an hour, the low intensity lamp giving an intensity of illumination of 1.8 ft. candles. At an instant noted with a stop-watch the light was put out, and the culture allowed to remain in darkness for a measured interval. The 150 watt lamp, giving an intensity of 40 ft. candles, was now pulled into position and flashed on for 0.10 minute. Meanwhile and during the ensuing darkness the position of the sporangiophore, read to the sharp upper meniscus of the sporangium, was observed at intervals of $\frac{1}{4}$ minute. These micrometer readings when plotted against the time follow a nearly linear or slightly declining sequence until the growth response begins, when the points rise rapidly (Fig. 1). The first significant upward deviation, read to the nearest quarter of a minute, is taken as the time of the response.

The sporangiophore is then readapted to the low intensity by an exposure of 30 minutes, and restimulated after different times in the dark with the same quantity of light energy.

III.

Table I presents the average data for the reaction times of twenty sporangiophores, each figure being the mean of ten separate deter-

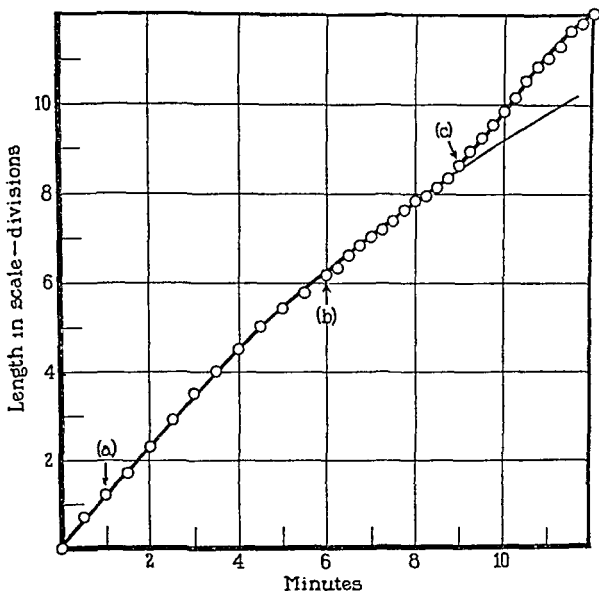


FIG. 1. The light growth response of *Phycomyces*. Sporangiophore previously growing in light of 1.8 ft. candles intensity, which is put out at (a). Dark adaptation of 5 minutes duration from (a) to (b). At (b) stimulation for 0.10 minute with 40 ft. candles. First increase in growth rate at (c), 3.0 minutes after stimulation. The slight falling off in rate during dark adaptation is not a random variation and usually occurs if the time in the dark is long enough. On the figure the trend of the growth curve previous to the response is continued as a thin line.

minations. Such averaging is justifiable, as seen from the small size of the corrected probable errors, in spite of the known individual

variability in absolute growth rate and in the temperature relations of growth (Castle, 1927-28). The photosensitive system must therefore possess a considerable degree of autonomy within the limits of the unicellular sporangiophore.

The reaction time clearly decreases in an orderly way with increasing time in the dark. Since stimulation in these experiments is effected with a constant quantity of light energy at constant intensity, the observed changes in reaction time are presumed to be due to changes in the concentration of a photosensitive substance which is

TABLE I.

Reaction Time during Dark Adaptation. Each Mean R. T. Is the Average of 10 Separate Determinations on a Number of Different Sporangiophores.

Time in dark	Mean R.T.	Probable error of the mean*
<i>min.</i>	<i>min.</i>	<i>min.</i>
2.0	3.58	±0.04
3.0	3.30	0.05
4.0	3.15	0.06
5.0	2.85	0.08
7.0	2.68	0.05
9.0	2.55	0.06
12.0	2.48	0.05
16.0	2.35	0.05
25.0	2.33	0.04

* Corrected for sampling by the method of Student (see Shewhart, 1926).

formed in the dark. The reaction time is therefore an inverse measure of the concentration of this substance, S , or

$$S = \frac{1}{R.T. - M}$$

where M is a constant representing the sum of the true latent period resulting as a consequence of photochemical action and any mechanical "action time" which may be present.

If the formation of S in the dark were a first order process, a plot of $\log (R. T. - M)$ against time in the dark should give a linear relationship. M is taken as 2.30 minutes, a value which is most favorable, within the limits of choice, to the log plot. The linear relationship

does not hold, as is seen in Fig. 2, where the points fall on a curved line, perhaps with an inflection point.

Assuming $M = 2.10$ minutes and $R.T._{(t=0)} = 7.4$ minutes, values

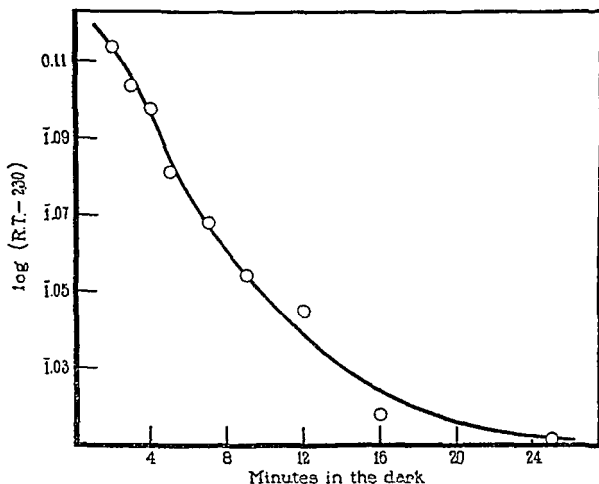


FIG. 2. Plot of the logarithm of the reaction time minus true latent period and "action time" against time in the dark. A linear relation would be expected if dark adaptation were a first order process.

of K have been calculated for pairs of adjacent points according to the formula for a bimolecular reaction

$$K(t_2 - t_1) a = \frac{x_2}{a - x_2} - \frac{x_1}{a - x_1}$$

where x stands for the amount of S formed. The average value of K is 0.012, and has been used to calculate the theoretical hyperbola

drawn through the observed points in Fig. 3 (a), by means of the formula

$$K = \frac{1}{at} \cdot \frac{x}{a - x}$$

The fit is reasonably good, and the course of dark adaptation therefore approximates the curve of a second order or "bimolecular" reaction.

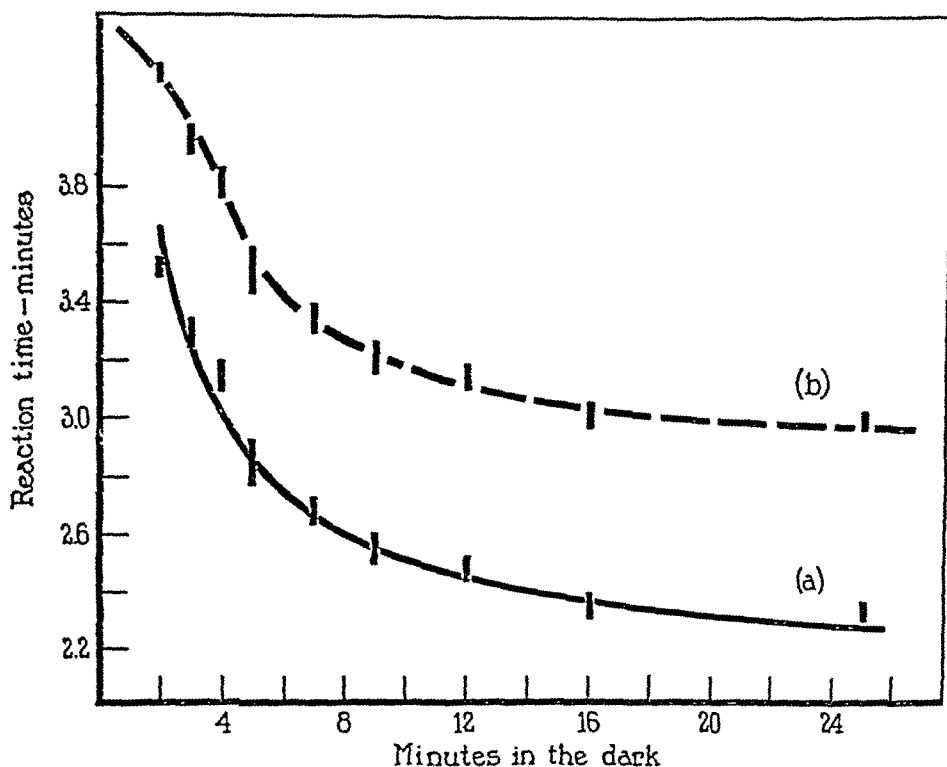


FIG. 3. Reaction time as a function of dark adaptation. In (a) the observed points are fitted with a hyperbola calculated according to the formula for a second order process. In (b) a free-hand curve is drawn through the same points, displaced on the ordinate scale, to illustrate the possible inflection of the curve. In each case the probable error of the mean reaction time is represented by one half the height of the symbol.

Crozier and Wolf (1928-29) studied the dark adaptation of *Agriolimnax* during continuous photic excitation, and found the equations of a second order process with autocatalysis by the product of the reaction

necessary to account for the kinetics of the "dark" reaction. It is possible that an initial lag and subsequent inflection point in the curve relating reaction time and time in the dark such as would be expected on the basis of autocatalysis exists in the course of the dark adaptation of *Phycomyces* (see Fig. 3 (b) and Fig. 2). The location of the inflection point is at least not contradictory to the autocatalytic hypothesis, although a final decision as to the presence or absence of autocatalysis

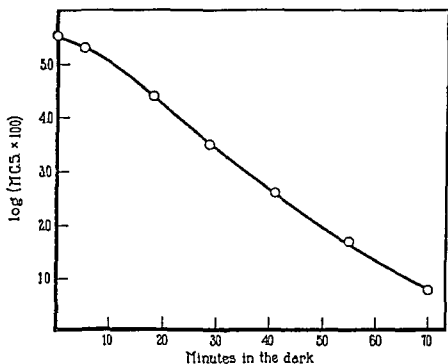


FIG. 4. Plot from the data of Tollenaar and Blaauw (1921) of the energy necessary to produce a just perceptible increase in growth rate after different times in the dark. (The time and intensity components of the energy product are not given in the original.)

cannot be made with the existing measurements. Another method of study of the photic adaptations of *Phycomyces* is at hand which is expected to render the distinction possible.

IV.

The only other attempt at a quantitative study of dark adaptation in plants has been made by Tollenaar and Blaauw (1921) on *Phycomyces nitens*. They determined the threshold sensitivity of sporangia

giophores by estimating the *quantity* of light necessary to produce a just perceptible increase in growth rate, following varying lengths of time in darkness after complete adaptation to light of 64 meter candles intensity. The logarithms of the energy products required to stimulate after different times in darkness are plotted in Fig. 4, taken from the data of Blaauw and Tollenaar, from which the authors conclude that the increase of sensitivity in the dark is a simple logarithmic function of the time in darkness, and that this relation "resembles the law of Weber for the comparison of two intensities."

This experiment rests on the assumption of the validity of the Roscoe-Bunsen law for the light-growth response, when the exposure time is varied over a considerable range. It must be emphasized that although this relation has been found to hold satisfactorily for minimal stimulation by light in the case of *Mya* (Hecht, 1919-20, *a*) the presence or absence of the response in this animal is a true criterion of threshold excitation, whereas in the light-growth response of *Phycomyces* the "threshold" is merely the least increase in growth rate *perceptible to the observer*. We may therefore be dealing with a response which is far from minimal as concerns the sporangiophore itself. In other words, if the "light" reaction in *Phycomyces* is rapid, as qualitatively seems to be true, then light adaptation will take place during the exposure time, provided the latter is of sufficient duration. In such a case the reaction time should be unduly long, and the complete differential for the production of *S* would have to be the sum of the partial differentials for the "light" and "dark" reactions. The validity of the Roscoe-Bunsen law for particular circumstances would not mean that the effectiveness of the light was at all times equivalent.

If we should assume that the curve in Fig. 4 represents the unopposed course of the "dark" reaction which regenerates *S*, the sensitive substance, it may be significant that there is an early inflection point as demanded by the notion of autocatalysis, and that the subsequent points do not fall on a straight line. In any case, the logarithmic relationship which might hold were Weber's law to be followed clearly does not exist, and since the data of Tollenaar and Blaauw give neither time nor intensity it is impossible to make with surety any quantitative inferences from the shape of the curve.

V.

It seems significant that the kinetics of dark adaptation in the unicellular sporangiophores of *Phycomyces*, where the mechanical aspects of the light response are so different from those in other forms, should prove to be strictly comparable to the other photosensory systems which have been studied, all of the latter being in animals: *Mya* (Hecht, 1918-19), the human retina (Hecht, 1919-20, b), and *Agriolimnax* (Crozier and Wolf, 1928-29). In all of these systems the "dark" reaction which regenerates the sensitive substance is bimolecular, in spite of known differences in the specific nature of the photosensitive materials themselves. This similarity points to a certain identity in the *kind* of chemical mechanics that underlies the sensitivity of organisms to light.

SUMMARY.

1. A single-celled, elongating sporangiophore of *Phycomyces* responds to a sufficient increase in intensity of illumination by a brief increase in growth rate. This is the "light-growth response" of Blaauw.

2. The reaction time is compound, consisting of an exposure period and a latent period (this comprising both the true latent period resulting from photochemical action and any "action time" necessary for the response). During the latter period the plant may be in darkness, responding nevertheless at the end of the latent period.

3. Both light adaptation and dark adaptation occur in the sporangiophore. The kinetics of dark adaptation can be accounted for on the basis of a bimolecular reaction, perhaps modified by autocatalysis. Attention is called to the bimolecular nature of the "dark" reaction in all other photosensory systems that have been studied, in spite of the diversity of the photosensitive substances themselves and of the different forms of the responses to light.

I wish to acknowledge with thanks the suggestion by Dr. W. H. Weston, Jr. of the suitability of *Phycomyces blakesleanus* for the purposes of these experiments.

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HEME AND TISSUE IRON.

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(Accepted for publication, October 4, 1928.)

There are a variety of heavy metal compounds in living cells. These are supposed to be involved in the catalysis of biological oxidations. It is by combination with these heavy metal compounds that the respiratory poisons cyanide, hydrogen sulfide, and carbon monoxide are supposed to act.* Little has been known about the precise nature of these substances except that one cannot get a test for free ionic iron. Yet the properties of an iron compound, depend very much on the nature of the complex of which the iron is a part. Thus the iron in hemoglobin has not the properties of the iron in ferric chloride. Over 40 years ago, however, MacMunn (10) observed in a great variety of animal tissues a pigment complex related to heme, the iron pyrrol part of hemoglobin. Recently Keilin (6) has shown that this complex, which he calls cytochrome, is present in the aerobic tissues of both plants and animals generally and that it consists of a mixture of pigments allied to hemochromogen. The components of cytochrome are not identical with the hemochromogen prepared from hemoglobin. There are likewise other naturally occurring pigments, chlorocruorin, heliocorubin, and actineohematin, which either are or can be converted into pigments similar to but not identical with the hemochromogen from hemoglobin.

The old view of hemochromogen was that it is simply reduced heme, the iron pyrrol complex of hemoglobin. On the basis of this view the various pigments which have been mentioned all contain different iron pyrrol complexes since they differ from each other and from the

*The reactions between the heme pigments and the specific inhibitors of respiration will be discussed in a later paper.

hemochromogen of hemoglobin. The writers (1, 3) have shown, however, that every hemochromogen consists of heme joined to some nitrogenous substance. The possibility thus arises that the various different hemochromogen-like pigments contain the same heme joined to different nitrogenous substances. When the unknown nitrogenous substances of heliocorubin and actineohematin were replaced with pyridine or ammonia the resulting hemochromogens were identical spectroscopically with the pyridine or ammonia hemochromogens prepared from the heme of hemoglobin. So we concluded (2) that heliocorubin and actineohematin contain heme. By the same method Fox (5) later showed that the iron pyrrol complex of chlorocruorin is different from heme. The addition of pyridine to yeast produced typical pyridine hemochromogen (2). This led us to suppose that cytochrome itself contained heme. But Keilin (7) has since presented evidence that there is in yeast in addition to cytochrome large amounts of invisible heme. Our pyridine hemochromogen may have come solely from this heme and not from cytochrome. This leaves the precise nature of the iron pyrrol nuclei of cytochrome and their relation to heme an open question. Nevertheless, the experiments based on the new view of the composition of hemochromogen permitted the identification of the heme of hemoglobin in aerobic tissues generally and they showed that in the study of the naturally occurring iron pyrrol pigments the ability of iron pyrrol groups to combine with nitrogenous substances must be taken into consideration. The significance of these results is emphasized by the recent experiments of Kuhn and Brann (9) and of Krebs (8) which show that the catalytic powers of heme are greatly changed when the heme is slightly altered or when it is combined with a nitrogenous substance.

The demonstration that heme itself is present in all animals makes less surprising the extraordinarily haphazard distribution of the hemoglobins. It would be interesting to know whether other substances containing metal pyrrol groups such as hemocyanin and chlorocruorin have, like the heme pigments, some ancestor common to all animals.

The fact that heme is universally distributed raises the question of how much of the iron of organisms is in the form of heme and how much in the form of still unknown iron compounds. In our original paper (2) we stated that we were attempting to answer this question

by parallel total iron and heme analyses, the heme being extracted with pyridine and estimated as pyridine hemochromogen. Our results have hitherto not been published because the validity of such a technique has become doubtful. In the meantime, however, several investigators (4, 11) have carried out heme estimations using essentially the same procedure we suggested. And von Euler and Fink (4) have come to the conclusion that, in the yeast they had, only about one 1/160 of the total iron is in the form of heme.

If pyridine and a reducer are added to yeast and the mixture first shaken and then centrifuged there is obtained on top a clear pyridine solution and on bottom a layer of yeast. When examined spectroscopically both these layers show hemochromogen-like bands. In the

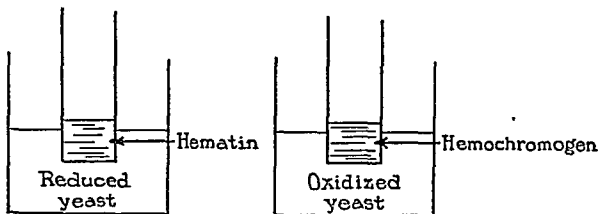


FIG. 1.

analyses of von Euler and Fink the pigment was examined in the clear pyridine solution and the assumption made that the concentration was the same in the yeast layer which was not examined. Such analyses are of little value in the absence of any precise information about the distribution of heme between the two layers. As a matter of fact, in the two cases we have studied, baker's yeast (Fleischmann's) and the wing muscles of bees, the assumption of equal distribution is not even approximately correct; mere inspection shows the concentration of hemochromogen to be much greater in the neglected yeast layer than in the clear pyridine solution.

Since we were unsuccessful in obtaining anything approaching a complete extraction of heme we decided to estimate the hemochromogen by comparing the density of its α band in the complete yeast-

pyridine mixture with the density of the α band of a series of known solutions of pyridine hemochromogen. In pure hemochromogen solutions whose concentrations are 10 per cent different one can readily detect the difference in the intensity of the bands. The hemochromogen from hemoglobin can be substituted for pyridine hemochromogen. If the heme contents of the solution of these two hemochromogens are the same, the intensity of the α bands are about the same too. In these experiments there were added to 4 gm. of moist yeast 1.5 cc. of $1/5$ N NaOH and 1 cc. of pyridine.

It is necessary to compensate for the absorption of the yeast and its pigments. That is done as shown in the figure. By "oxidized yeast" (*cf.* Fig. 1) is meant yeast shaken with air for 5 minutes, which procedure causes the disappearance of the hemochromogen bands.

This method of analyses gives the result that 40 ± 10 per cent of the total iron of baker's yeast is in the form of heme. The total iron is 0.07 mg. per gm. dry weight.

One cannot tell how much of the iron of yeast has been introduced as an impurity during the commercial preparation of the yeast. This difficulty is avoided when the wing muscles of bees are used. Furthermore, the great activity of these muscles—they can contract several hundred times a second—is associated with an iron content even 2.8 times as great as that of yeast, namely, 0.196 mg. per gm. dry weight. Despite this great difference in the total iron we again found about 40 per cent of the iron to be in the form of heme.

The muscles were removed from frozen bees by the ingenious technique devised by Keilin (6). Care was taken not to introduce extraneous iron and to make the removal of the muscles as uniform as possible. Batches of the muscles from ten bees gave uniform analytical results.

The great weakness of these analyses lies in the use of pyridine hemochromogen as a standard. As Keilin (7) has pointed out, the hemochromogen-like substances obtained from cytochrome itself when pyridine is added may not be identical with pyridine hemochromogen. Furthermore, the α band of the hemochromogen seen in the muscle tissue obtained on centrifuging the muscle-pyridine mixture is not in precisely the same position as the α band of pyridine hemochromogen. These uncertainties render doubtful the estimations of heme based on the pyridine technique in any of its forms.

We hope, nevertheless, that the discussion and experiments which have been presented bring out some of the difficulties which have to be faced, and that they make clear, at least, that there is at present no adequate experimental basis for the view that only a very small part of tissue iron is in the form of heme. The evidence given by the type of procedure described in this paper points to heme being an important form of tissue iron.

The pyridine extract of yeast contains in addition to a hemochromogen similar to pyridine hemochromogen another hemochromogen-like pigment (2). Whether this second pigment contains an iron pyrrol complex is not known. There is the possibility that some and perhaps all of the iron not in the form of heme may exist in other iron pyrrol complexes.

SUMMARY.

1. A method is described for estimating the heme in yeast and bees' muscles as pyridine hemochromogen.
2. The difficulties of the method are discussed.
3. The heme as given by the pyridine method is responsible for about 40 per cent of the total iron.

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SPECTROPHOTOMETRIC STUDIES OF PENETRATION.

V. RESEMBLANCES BETWEEN THE LIVING CELL AND AN ARTIFICIAL SYSTEM IN ABSORBING METHYLENE BLUE AND TRIMETHYL THIONINE.

By MARIAN IRWIN.

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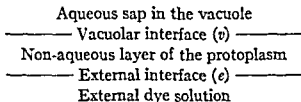
(Accepted for publication, June 15, 1928.)

I.

INTRODUCTION.

This paper deals with an attempt to imitate the action of the living cell in respect to penetration of dyes.

The protoplasm of a living cell probably consists of two non-aqueous layers¹ (one in contact with the external medium and the other with the vacuolar sap), which are separated by an aqueous middle layer. But in setting up an artificial system we may confine ourselves to the simple case where the rate of diffusion appears to be controlled by three phases, as if a living cell consisted of only one non-aqueous layer lying between the external solution and the aqueous sap of the vacuole, as shown by the following diagram:



¹ We are unable to say which layer plays the more important rôle in regulating the rate of penetration into the vacuole. The controlling factor may change with alterations in the condition of the cells brought about by experiments. It seems possible that the external non-aqueous layer is more polar than the vacuolar layer, in that the ions penetrate the former more rapidly than the latter. In the case cited in the text the vacuolar non-aqueous layer may be responsible for the control of the rate. But in any case it seems probable that the net result may be treated as if the rate were controlled by a system containing only one non-aqueous layer.

In such a system the diffusion of a dye will be greatly affected by the partition coefficients:

$$K_e = \frac{\text{Concentration of dye in the non-aqueous layer}}{\text{Concentration of dye in the external solution}}$$

$$K_v = \frac{\text{Concentration of dye in the non-aqueous layer}}{\text{Concentration of dye in the aqueous sap}}$$

The higher K_e is, the more rapid is the rate of diffusion from the external solution through non-aqueous layer. On the other hand, the lower K_v is, the more rapid is the rate of diffusion through the non-aqueous layer. The values of K_e and K_v and concentrations of dye in the aqueous solutions will determine the diffusion gradient in the non-aqueous layer.

In the case of methylene blue, diffusion into the vacuole from the external solution is very slow because K_e is so small that though K_v is low there is very little dye in the non-aqueous layer² to diffuse into the sap. When a dye exists in two forms, with free base predominating at higher pH values and salt predominating at lower pH values, it is necessary to consider the partition coefficient of each form of dye at each interface. In the case of azure B the partition coefficient of the dye in the form of free base,³ K_{eb} , is so high that its diffusion through

² According to Overton's theory penetration depends on the solubility of the dye in the lipid layer of the living cell, which involves only one partition coefficient, K_p . This is sufficient to account for the lack of penetration of dyes which are insoluble in lipid and for the rapid penetration of some of the dyes soluble in lipid, but does not explain why a dye like crystal violet, which is very soluble in lipid, does not readily penetrate the cell. On the basis of the theory presented in the text the slow penetration of crystal violet into the cell is explainable; though the dye penetrates the non-aqueous layer it does not enter the vacuole rapidly on account of K_v being so high.

³ Though the dye in form of free base is often considered to be undissociated, this is an open question. The free base may be dissociated just as much as the dye in form of salt. We must therefore leave the question of the extent of dissociation of the "free base" for future solution by organic chemists, while we may conclude with more certainty that the "salt" is dissociated. This "free base" must be distinguished from the "pseudo base" which exists at still higher pH values. "Free base" may possibly be represented by an anhydro-base, or by a structure like the salt except that the halide is replaced by the hydroxyl group. (Irwin, M., *J. Gen. Physiol.*, 1926-27, x, 927 and 928, foot-note 3.)

non-aqueous layer from the external solution is rapid. But even if K_{sb} (the partition coefficient of the same form of dye at the vacuolar interface) is high, the dye readily diffuses through the non-aqueous layer into the vacuole because it is transformed to salt as soon as it comes in contact with the sap (on account of the low pH value of the sap) and this greatly increases the diffusion gradient. Since the partition coefficient of the dye in the form of salt at the vacuolar interface, K_{ss} , is low, its backward diffusion from the vacuole through non-aqueous layer is very slow. The dye salt readily collects in the vacuole. The same type of behavior occurs if a dye enters into combination with the sap to form a very slightly soluble compound.

This view would explain why azure B penetrates more rapidly than methylene blue into the vacuole of a living cell, as described in previous papers,⁴⁻⁶ and would lead us to predict that the same thing would happen with an artificial system consisting of (1) the same dye solutions as those employed for the living cells, (2) chloroform to represent the non-aqueous layer of the protoplasm, and (3) sap freshly extracted from the vacuoles of living cells or artificial sap. Since the non-aqueous part of the protoplasm is but crudely represented by chloroform, the system may bear only a qualitative resemblance to the living cell.

If this theory⁷ be correct we shall expect that when mixtures of dyes are employed the order of penetration will be the same in the artificial system as in the living cell.

II.

Methylene Blue.

A. Comparison of the Artificial System with Valonia.

Previous analyses^{5,6} have shown that the vacuole of *Valonia* takes up azure B from a solution of methylene blue in sea water at pH 9.5. In order to compare this with the artificial system two types of

⁴ Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, xxiv, 425.

⁵ Irwin, M., *J. Gen. Physiol.*, 1926-27, x, 927.

⁶ Irwin, M., *J. Gen. Physiol.*, 1928-29, xii, 147.

⁷ This theory has been successfully tested in the case of many dyes (Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 127; 1928, xxvi, 125).

experiments were made. (1) One experiment is to show the absorptive power of the chloroform in relation to the aqueous dye solution (representing the external dye solution used for living cells). This is done by first shaking the chloroform with the aqueous dye solution, allowing the chloroform to evaporate, and then dissolving the dye residue in the sap or in distilled water. The dye thus obtained is called for convenience "the dye obtained from chloroform by evaporation."

(2) The second experiment is to determine how readily the chloroform is able to give up the dye to the sap. This is done by first shaking the chloroform with the aqueous dye solution and subsequently extracting the dye from the chloroform by shaking it up with the sap.

If chloroform adequately represents the non-aqueous layer of a living cell we may by studying the behavior of the chloroform⁸ toward the dye in these two aqueous phases learn something of the behavior of the non-aqueous layer of the cell in relation to phases inside and outside the cell.

The experiments were therefore carried out as follows.

200 cc. of solution of methylene blue (French) dissolved in sea water at pH 9.5 or at pH 5.5 was shaken up with 50 cc. of pure chloroform in a separatory funnel; the chloroform was then removed from the funnel and if it contained even a trace of water in the form of droplets the latter was removed by decanting the chloroform repeatedly from one beaker to another; the drops of water then adhered to the wall so that eventually chloroform free from drops of water could be obtained by this method. The chloroform was then divided into two equal parts: one portion was allowed to evaporate and the dye residue was dissolved in freshly extracted sap of *Valonia* (the sap contained about 0.6 M halides and had a pH value of about 5.8) or in distilled water. The second portion was shaken with sap until a concentration sufficient for spectrophotometric analysis was obtained. The sap was separated from the chloroform by pipetting it off (there was not sufficient chloroform in the sap to affect the absorption spectra of the dye in the sap).

The sample of methylene blue (French) gave an absorption curve with primary absorption maximum at 664 m μ when dissolved in sea water at pH 9.5 or pH 5.5 (Fig. 1, symbol \square), which is characteristic of a dye solution consisting chiefly of methylene blue.

⁸ Though the non-aqueous layer is represented by the chloroform, it does not signify that it resembles chloroform in chemical composition. It is uncertain as to whether the non-aqueous layers are "lipoid," but their behavior suggests it. The question of the character of the non-aqueous layers must therefore be left undecided for the present.

With this sample of methylene blue in sea water at pH 9.5, the dye (1) obtained from chloroform by evaporation, and (2) extracted from the chloroform by shaking with sap, gave an absorption curve characteristic of a dye consisting chiefly of azure B (with primary absorption

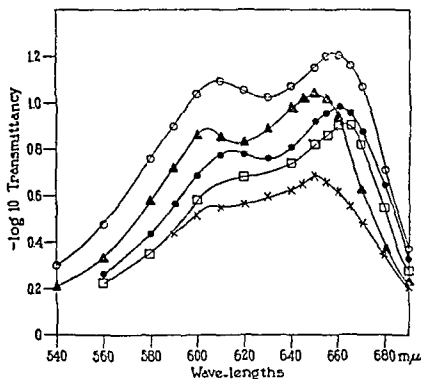


FIG. 1. Absorption curves obtained in experiments on methylene blue (French). (Comparison with *Valonia*.) Symbol \square represents the methylene blue dissolved in sea water at pH 5.5 or pH 9.5. Symbol \blacktriangle represents the dye taken up by chloroform from methylene blue in sea water at pH 9.5, then freed from chloroform by evaporation, and finally dissolved in distilled water. Symbol \times represents the dye extracted by sap of *Valonia* from chloroform which had previously been shaken with methylene blue in sea water at pH 9.5. Symbol \circ represents the dye taken up by chloroform from methylene blue in sea water at pH 5.5, then freed from chloroform by evaporation, and finally dissolved in distilled water. Symbol \bullet represents the dye extracted by the sap of *Valonia* from chloroform which had previously been shaken with methylene blue in sea water at pH 5.5. The measurements were made on a layer 1 cm. thick.

maximum at 650 m μ ; Fig. 1, symbols \blacktriangle and \times), which readily enters the chloroform because at pH 9.5 it is largely in the form of free base; it readily comes out into sap because the acidity of the sap changes it to dye salt which has a low value of K_v . The reddish violet of the dye in chloroform may indicate the absorption chiefly of azure B in the

form of free base and a smaller amount of azure B in form of salt and methylene blue. With the solution at pH 5.5 the dye obtained from chloroform by evaporation gave a primary absorption maximum at 658 $m\mu$ (Fig. 1, symbol ○) and the dye extracted by the sap from the chloroform gave a primary absorption maximum at 660 $m\mu$ (Fig. 1, symbol ●), indicating that in both cases there was a mixture of azure B and methylene blue (the former containing slightly less methylene blue than the latter). The color of the dye in chloroform was blue, thus showing that if azure B was absorbed it was in form of salt. The amount of azure B in form of salt and of methylene blue absorbed is less than in the case of azure B in form of free base.

The result obtained at pH 9.5 is in complete agreement with that obtained with living cells of *Valonia* (uninjured). Unfortunately it is not possible to compare the result with that of the living cell in solutions at pH 5.5 since in the latter case penetration is so slow that analysis is not possible at present. But if the present theory be correct we might expect a mixture of these two dyes at pH 5.5 to penetrate the cell but only more slowly than azure B from the solution at pH 9.5.

B. Comparison of Nitella flexilis with the Artificial System.

Previous experiments⁹ have shown that from methylene blue solution (French or Merck's medicinal) at pH 9.2 azure B is readily taken up by the vacuole of living cells of *Nitella*. For comparison with the behavior of an artificial system the following experiments were carried out.

The sample of methylene blue (Merck's medicinal) dissolved in buffer solution at pH 9.2 or pH 5.5 gave an absorption curve characteristic of methylene blue (Fig. 2, symbol ■).

The same technique as described under *A* was employed (1) for absorption of dye by chloroform from this sample of methylene blue dissolved in diluted buffer solution at pH 9.2 and pH 5.5, and (2) for the extraction of dye from the chloroform by the artificial sap of *Nitella*. It was not possible to use the real sap

⁹ In 1926-27 preliminary statements of these results on *Nitella* were made on page 426 of the paper referred to in foot-note 4, and on page 945 of the paper referred to in foot-note 5. In 1928 these results in detail were reported in the paper referred to in foot-note 6, and in *Proc. Soc. Exp. Biol. and Med.*, 1927, xxv, 563.

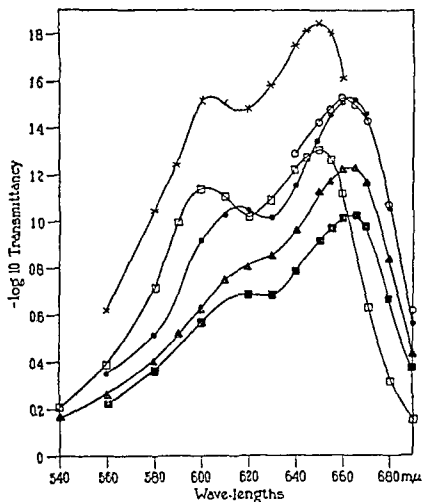


FIG. 2. Absorption curves obtained in experiments on methylene blue solution (Merck's medicinal). (Comparison with *Nitella*.) Symbol ■ represents methylene blue solution at pH 9.2 or at pH 5.5. Symbol □ represents the dye absorbed by chloroform from methylene blue solution at pH 9.2, freed from chloroform by evaporation, and then dissolved in artificial sap of *Nitella*. Symbol × represents the dye extracted by artificial sap of *Nitella* from chloroform which has absorbed the dye from methylene blue solution at pH 9.2. Symbol ○ represents the dye absorbed by chloroform from methylene blue solution at pH 5.5. The chloroform was allowed to evaporate and the dye residue was dissolved in artificial sap of *Nitella*. Symbol ● represents the dye extracted by artificial sap of *Nitella* from chloroform previously shaken with methylene blue solution at pH 5.5. Symbol ▲ represents the dye extracted with distilled water at pH 5.5 from chloroform previously shaken with methylene blue solution at pH 5.5. The curves with the symbols ●, ■, × refer to measurements made with a layer 1 cm. thick, the others to measurements made with a layer 0.3 cm. thick.

of *Nitella* because the protein coagulates on shaking. The artificial sap was therefore made up with 0.11 M KCl dissolved in a solution of M/150 phosphate buffer at pH 5.5. All measurements described in the text were made immediately after extractions.

With the solution at pH 9.2 the dye obtained from chloroform by evaporation or by extraction with the sap gave an absorption curve characteristic of a dye containing chiefly azure B with a primary ab-

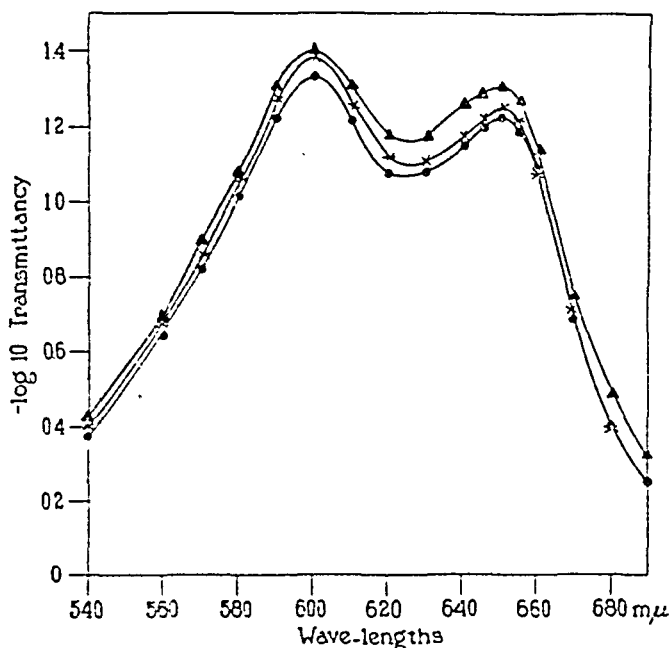


FIG. 3. Absorption curves obtained in experiments on azure B or trimethyl thionine (Holmes) in sea water. (Comparison with *Valonia*.) Symbol \times represents the azure B dissolved in sea water at pH 9.5 or pH 5.5. Symbol \bullet represents the dye extracted by sap of *Valonia* from chloroform shaken with azure B in sea water at pH 9.5; symbol \blacktriangle the same at pH 5.5. The measurements were made on a layer 0.6 cm. thick.

sorption maximum at 650 mμ (Fig. 2, symbol \square and \times). The color of the dye in chloroform was violet red thus indicating that azure B in the form of free base was absorbed. But with the solution at pH 5.5 the dye obtained from chloroform by evaporation gave a primary absorption maximum at 660 mμ (Fig. 2, symbol \circ); the dye extracted by the sap from the chloroform gave a primary absorption maximum at 663 mμ (Fig. 2, symbol \bullet), indicating that a mixture containing chiefly

methylene blue with some azure B was absorbed by the chloroform, but mostly methylene blue was extracted from the chloroform by the sap. The color of the dye in chloroform was blue, thus showing that some azure B in form of salt or methylene blue was absorbed.

When the experiments were repeated at pH 9.2 with methylene blue (French) the same result was obtained as with Merck's medicinal.

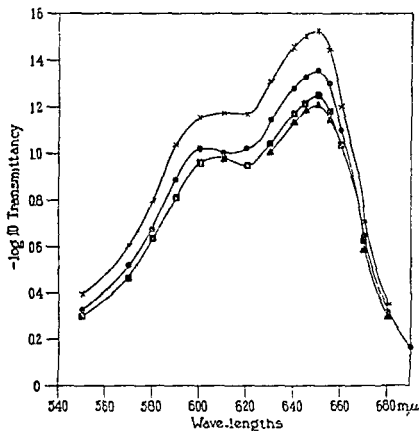


FIG. 4. Absorption curves obtained from experiments on azure B (Holmes) dissolved in buffer solutions. (Comparison with *Nitella*.) Symbol ■ represents the dye dissolved in buffer solution at pH 9.2 or at pH 5.5. Symbol × represents the dye absorbed by chloroform from azure B solution at pH 9.2. The dye was freed from chloroform by evaporation and dissolved in distilled water. Symbol ● represents the dye extracted by artificial sap of *Nitella* from chloroform shaken with azure B solution at pH 9.2; symbol ▲ the same at pH 5.5. The measurements were made on a layer 0.6 cm. thick.

The results obtained with methylene blue at a pH value a little above 9 were in general agreement with the experiments on living cells (uninjured). At pH 5.5, the comparison is not possible since the penetration is so slow that there is not sufficient dye collected for analysis in the sap before there was a possibility of injury to the cell.

III.

Azure B or Trimethyl Thionine.

Using the method described in section II experiments were repeated with azure B for comparison of the artificial system with *Valonia* (Fig. 3) and with *Nitella* (Fig. 4). In both cases the dye absorbed by chloroform and set free by evaporation or extracted by the sap from chloroform gave the absorption curve of a dye consisting chiefly of azure B with a primary absorption maximum at $650\text{ m}\mu$ (Fig. 3, symbols \bullet and \blacktriangle and Fig. 4, symbols \times , \bullet , and \blacktriangle), which is identical with the dye solutions in which chloroform was shaken up (Fig. 3, symbol \times and Fig. 4, symbol \blacksquare). These results are in exact agreement with those obtained with uninjured cells of *Valonia* and of *Nitella*.

IV.

CONCLUSION.

The rate of diffusion¹⁰ through the non-aqueous layer of the protoplasm depends largely on the partition coefficients mentioned above. Since these cannot be determined we have employed an artificial system in which chloroform is used in place of the non-aqueous layer of the protoplasm. The partition coefficients may be roughly determined by shaking up the aqueous solutions with chloroform and analyzing with the spectrophotometer (which is necessary with methylene blue because we are dealing with mixtures). This will show what dyes may be expected to pass through the protoplasm into the vacuole in case it behaves like the artificial system.

From these results we may conclude that the artificial system and the living cell act almost alike toward methylene blue¹¹ and azure B, which supports the notion of non-aqueous layers in the protoplasm.

There is a close resemblance between *Valonia* and the artificial system in their behavior toward these dyes at pH 9.5.

¹⁰ Cf. Northrop, J. H., *J. Gen. Physiol.*, 1928-29, xii, 435.

¹¹ Methylene blue solution is generally found to contain azure B as impurity but in too small a concentration to affect the primary absorption maximum which is at about $665\text{ m}\mu$. It is, however, possible to determine roughly by extraction with chloroform the relative amount of azure B present in methylene blue solutions

In the case of *Nitella*, on the other hand, with methylene blue solution at pH 9.2 the sap in the artificial system takes up relatively more azure B (absorption maximum at 650 $m\mu$) than the vacuole of the living cell (655 $m\mu$). But both take up azure B much more rapidly than methylene blue.

A comparison cannot be made between the behavior of the artificial system and that of the living cell at pH 5.5 since in the latter case there arises a question of injury to cells before enough dye is collected in the sap for analysis.

since azure B in form of free base is absorbed by chloroform from methylene blue solution much more rapidly than methylene blue itself. Azure B may therefore be readily extracted from methylene blue solution at about pH 9. The dye thus extracted is freed from chloroform by evaporation and is dissolved in various aqueous solutions. The dye thus extracted contains various proportions of azure B and methylene blue giving primary absorption maxima from 650 $m\mu$ (that of the pure azure B) to 662 $m\mu$ (nearly that of purest available methylene blue), depending on the amount of azure B in proportion to methylene blue present in the methylene blue solution from which the extraction was made.

Another method of detection is by observing the color of the dye in chloroform since azure B in form of free base appears violet red, while methylene blue appears blue. But this method is inadequate if the extracted dye is a mixture or if the concentration is low where color matching becomes difficult.

At about pH 9 we find that the more azure B a methylene blue solution contains the more rapid is the rate of penetration of the blue dye into the vacuole of a living cell as well as into the artificial "vacuole."

If the penetration is very slow there are possibilities of errors arising from injury and contamination as already stated (Irwin, M., *J. Gen. Physiol.*, 1928-29, xii, 147) so that the sample "French" was chosen, which had a higher rate of penetration than any other samples (it contained more azure B than others). With *Valonia*, even with the sample "French," the rate of penetration was so slow that it could not very well have been lowered without rendering the results doubtful on account of the possibilities of error already stated. For this reason conclusive results were not possible from experiments with other samples which showed a slower rate of penetration. But with *Nitella* the penetration was more rapid with each of the samples employed so that it was possible to employ a greater variety of samples.

The analogy between chloroform and the living cell is only a rough one. The resemblance, for example, exists in so far as both systems (living and artificial) take up azure B much more rapidly than methylene blue but we need not suppose that the dyes absorbed by both systems will give exactly identical absorption curves in every instance.

These resemblances both in *Valonia* and in *Nitella* become less as injury increases on account of the increase in penetration of methylene blue into the vacuoles of injured cells.

CARBON DIOXIDE FROM THE UNSEVERED VAGUS NERVE OF THE SNAKE.

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In all previous tests for the carbon dioxide discharged by nerves these parts have been worked upon after they have been cut free from the animal serving as a source. It therefore seemed desirable to measure the output of this gas from a nerve whose normal relations to the animal were as little disturbed as possible, and whose central and peripheral ends at least were not severed from their connections. After several unsuccessful trials it was decided to attempt this work on the snake's vagus nerve which, in consequence of its considerable length, seemed favorable material for such an investigation. This research was carried out under a grant from the Bache Fund of the National Academy of Sciences. The writer wishes to express his obligation to the Directors of this Fund for the essential aid rendered.

At the outset of the work it was supposed that the vagus nerve of any small snake would serve for the problem at hand, but it was quickly found that even in the largest local snakes the vagus was much too delicate and too short to meet the needs of the experiment. Large snakes were therefore sought, and satisfactory material was finally found in the gopher-snake of Texas and Mexico, *Drymarchon corais couperi* (Holbrook). Snakes of this species were obtained alive from a local collector. They measured in general about two meters in length. They were restrained from undue movement by being placed in a long straight metal tube about 6 cm. in diameter. To manipulate them in the tube, cords were attached one to the snake's jaw and the other to its tail. As a preparatory step the head of the snake was drawn out of the tube for a convenient distance and held there. With a single blow a sharp blade was driven through the head including the brain which was cut through transversely just behind the cerebral

hemispheres thus reducing the animal to the decerebrate condition. An incision was then made along the left-ventral aspect of the body from a point at the base of the skull to the region of the heart. The left vagus nerve was thus laid bare and could be dissected free throughout its exposed length. It remained attached, however,

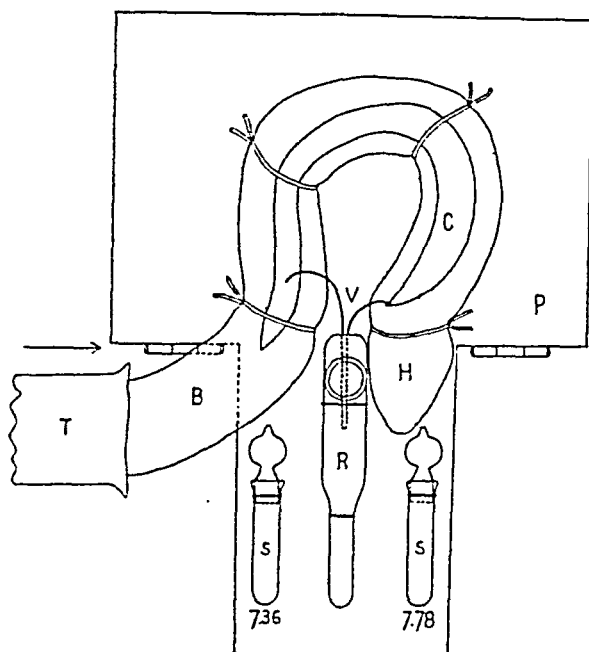


FIG. 1. Plan of the rocking platform and adjacent parts for the support of the anterior portion of the snake. *B*, body of the snake projecting from the metal tube; *C*, cut in the left side of the snake extending from the head to the heart; *H*, head of the snake; *P*, platform; *R*, respiratory chamber with the stoppered aperture uppermost; *S*, standard tubes pH 7.78 and pH 7.36; *T*, end of the metal tube in which the snake's body was restrained; *V*, vagus nerve looped through a small aperture into the respiratory chamber; its dotted portion corresponds to the outline of the glass rod on which it rests. The arrow on the side marks the axis around which the platform was rocked.

anteriorly to the brain and posteriorly to the region of the heart. In this way from 15 to 20 centimeters of free nerve could be made available.

The anterior portion of the snake over which the cut ran was laid out on a wooden platform (Fig. 1) in such a way as to bring the head and the heart of the animal as near as possible together. The portion

of the snake's body thus made into a loop was tied firmly to the supporting platform and the left vagus nerve already freed was looped in the opposite direction. The free loop of nerve was inserted into the respiratory chamber through an aperture at its end and this aperture was then closed completely by the mixture of kaolin and vasoline already described for this purpose (Parker, 1925, p. 649). Inside the respiratory chamber the loop of nerve rested on a glass rod which ran lengthwise through the larger section of the chamber and close to its axis (Fig. 1).

The respiratory chamber used in these experiments was a tube of pyrex glass much like that employed in my earlier work. It had a capacity of 42 cc. (5 cc. for fluid and 37 cc. for air), and required 0.00686 mgm. of carbon dioxide to change its contents from pH 7.78 to pH 7.36 (Parker, 1925, 1928). The chief differences between the type of chamber used in these tests and that used in the earlier series concerned the apertures. In the present chamber the principal aperture was an opening on the upper side of the tube directly above the central glass rod on which the nerve was placed. Through this opening the nerve could be manipulated and brought into appropriate position. This opening was closed by a tightly fitting ground-glass stopper. In addition to this there were two other openings both at the large end of the chamber. One of these was just above the central glass rod and served for the insertion of the loop of nerve. The other was to one side and was used for the insertion of a capillary glass tube by means of which a current of pure air could be bubbled through the solution in the chamber thus freeing both it and the chamber of carbon dioxide. The two apertures could be closed with the mixture of kaolin and vasoline already described. As in the earlier experiments the respiratory chamber had next to it on the platform a pair of tubes, carrying standard indicator solutions one at pH 7.78, the other at pH 7.36.

It usually required from two to two and a half hours to prepare the vagus nerve of a gopher-snake for testing. By the end of this time the period of gush had already passed, if in fact there had been one, and the first possible reading must have represented the steady outflow of carbon dioxide. After the loop of nerve had been put in place the capillary tube was introduced through its special aperture and

sufficient pure air was bubbled through the liquid in the narrow part of the chamber to bring the chamber to a point well on the alkaline side of pH 7.78. The whole platform was then put in rocking motion as already described (Parker, 1925, p. 645). When the color of the indicator in the respiratory chamber agreed with that of the standard tube pH 7.78 a stop-watch was started, and when it later coincided with that in the other standard tube pH 7.36 the watch was stopped.

TABLE I.

Carbon dioxide in milligrams per gram of nerve per minute given out by the vagus nerve of the gopher-snake, *Drymarchon corais couperi* (Holbrook). The snakes are numbered I to VI and their lengths in centimeters are given in the second column of the table. The weight in milligrams of each vagus nerve tested is given in the third column. On the vagus nerve of each snake three or four readings for carbon dioxide were made; these are recorded in the body of the table together with their averages. In each series the last reading was taken after the nerve previously attached anteriorly and posteriorly to the snake had had these attachments severed. Consequently the records to the left of the double vertical line in the table represent records from nerves whose proximal and distal termination were still intact while those to the right of this line represent records taken after these nerves had been cut free.

No.	Length cm.	Wt. mgm.	CO ₂ mgm. per gm. per min.				Averages
I	192	127	.0027	.0026	.0028	.0025	.00263
II	206	100		.0020	.0019	.0017	.00208
III	187	96		.0026	.0025	.0022	.00243
IV	198	112	.0031	.0020	.0022	.0018	.00228
V	173	103	.0026	.0026	.0018	.0020	.00225
VI	213	116		.0032	.0023	.0021	.00253
General averages00250	.00225	.00205	.00234

The interval of time thus measured represented the period over which the nerve gave out 0.00686 mgm. of carbon dioxide. At the close of an experiment the chamber could be again aspirated with pure air and a second reading taken. In this way it was often possible to get three or four readings from one nerve. At the end of the series the nerve was cut free and weighed. The weight of the nerve and the time required to change the contents of the chamber from pH 7.78 to pH 7.36 having been determined, the rate of discharge of carbon dioxide per gram of nerve per minute could be readily calculated.

Such rates are given in Table I for the vagus nerves of six snakes tested as described. These were the satisfactory trials out of some twenty attempts, the failures being due chiefly to movements of the snake whereby the nerves were dragged out of the respiratory chamber and in some cases torn free at one end or the other. No records were regarded satisfactory unless the heart of the snake was known to have been in continuous action during the test and had been brought to a standstill by an electric stimulus applied to the nerve close to where it emerged from the head of the snake. An inspection of Table I shows that the highest rate of carbon dioxide discharge for this nerve was 0.0032 mgm., the lowest 0.0017 mgm. of gas per gram of nerve per minute. The general average was 0.00234 mgm. This average is very close to that of my earlier determination for the lateral-line nerve of the dogfish, 0.0024 mgm., and only a little higher than that of Fenn (1927) for the same nerve, 0.0020 mgm.

The readings on which the snake determinations were based were taken in the late winter when the snakes were still in a semi-torpid condition. They are therefore more properly compared with the winter rates than with the summer rates for frogs. The average winter rate for the nerves from the large southern frog, *Rana grylio*, was found by me to be 0.0022 mgm., the summer rate 0.0034 mgm. of carbon dioxide per gram of nerve per minute. The agreement in this instance between the winter rate of the frog and of the snake is obvious, but it must be kept in mind that the rates for frog nerves are by no means settled. Fenn (1928) has recorded for the frogs on which he worked relatively high rates, averaging 0.0031 mgm. of carbon dioxide and Gerard (1927) has noted unusually low rates which, if transposed to the terminology of this paper, range from 0.00051 mgm. to 0.00038 mgm. of carbon dioxide per gram of nerve per minute. With Gerard's work carried out at 14°C. and Fenn's and mine at 22°C. it is by no means easy to draw satisfactory comparisons. Subsequent work may settle many of these differences. In the determinations on the snake nerve recorded in this paper all the tests were carried out in a room kept as near 22°C. as possible. It is a remarkable fact that nerves from frogs in the winter and in the summer condition, though tested in a room kept at a reasonably constant temperature, 22°C, nevertheless yield characteristically different results, showing that

notwithstanding the uniformity of the immediate surroundings the metabolic rates of the two classes of nerves are not to be confused.

The chief question in the present research was to determine whether there was a difference in the metabolic rates of nerves whose central and peripheral terminations were intact as contrasted with those in which such connections had been severed. The answer to this question seems to be in the negative. After two or more satisfactory readings had been obtained from the six snake nerves dealt with in this paper and the nerves had been shown by electrical stimulation to be in a condition to check the heart beat and thus to give evidence of normal vitality, they were as a last move cut free from all connections with the animal and a final reading for this state of isolation was taken. Such records are given in the next to the last column of Table I and their average is shown at the bottom of that column. This average, 0.00205 mgm., may be compared with the averages of the two preceding records, namely the immediately preceding one, 0.00225 mgm. and the next in advance of that, 0.00250 mgm. A comparison of these averages shows that the carbon dioxide from these nerves was gradually falling in amount and that this fall was not significantly changed by cutting the central and peripheral connections. In other words, the metabolism of resting nerve is a local activity uninfluenced by central and peripheral connections. It is therefore to be assumed that studies of this kind made on nerve removed from cold-blooded animals are as significant for an understanding of their metabolism as though the work had been carried out on nerves retaining their original connections.

In the instance under consideration it is probable that before the nerves were cut the regular beat of the heart gave evidence that normal nervous impulses were passing over the fibers. Nevertheless, when, by cutting the central and peripheral connections, the passage of these impulses was interrupted no observable change was noted in the carbon dioxide output. That there was such a change is highly probable but the fact that it was not observable probably means that it was a change which as compared with the total metabolism was too small to be noticed by the method here employed. I have estimated (Parker, 1925), that the method that I have used is sensitive to about five per cent of the total metabolism of the tissue studied. Changes that

proceed on less than this proportion would naturally escape detection. The normal activity of the vagus nerve seems to belong to this order and is without doubt well below that degree of activity which is excited when the nerve is artificially stimulated by induction currents. It is probable that in all the results of artificial stimulations as applied to nerve, the production of heat, the absorption of oxygen, and the discharge of carbon dioxide, these records are much in excess of any phase, even the extreme, that is exhibited by nerves under the natural conditions of bodily environment.

SUMMARY.

1. The vagus nerve of the gopher-snake, *Drymarchon corais couperi* (Holbrook), discharges carbon dioxide at an average rate of 0.00234 mgm. of gas per gram of nerve per minute. The extremes were 0.0032 mgm. and 0.0017 mgm.

2. These rates were maintained as well when the nerve was separated from its central and its peripheral connections as when these connections were intact showing that the resting metabolism of nerve is a local operation and is not immediately dependent upon distant connections.

3. These observations also point to the conclusion that the passage of normal impulses over a nerve call for an increase of activity that is unobservable by the method employed and that must be small in amount compared with that produced by artificial stimulation.

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THE PENETRATION OF STRONG ELECTROLYTES.

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This paper deals primarily with the penetration of strong electrolytes. It touches incidentally on the problem of flotation since in some cases flotation was used as a criterion of penetration: as examples of this we may cite experiments in which NH_4Cl and CsCl were added to sea water.

Striking results were obtained by adding small amounts of 0.6 M NH_4Cl to sea water. The specific gravity of the sea water was very slightly decreased and at first the cells remained at the bottom but after a time they began to float because NH_4Cl penetrated and lowered the specific gravity of the sap so much that the cells rose to the surface: here they continued to live and grow indefinitely. (It is probable that NH_3 or NH_4OH penetrates and is subsequently changed to NH_4Cl).

When the concentration of NH_4Cl in the sea water was 0.005 M² enough NH_4Cl penetrated in 10 days³ to cause the cells to float. Examination showed that the specific gravity of the sap was then lower than that of the sea water (see Table I); it was analyzed to determine the proportions of salts present and it was found that artificial solutions made up in these proportions had practically the same specific gravity as that of the samples of sap.⁴ Obviously therefore the penetration of NH_4Cl is sufficient to account for the change in specific gravity (see Table I).

¹ Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 234.

² This did not increase the osmotic pressure of the sea water more than 0.1 per cent.

³ These cells were placed in bottles and submerged in the ocean where growth continued.

⁴ The composition of these samples will be discussed in a later paper. The specific gravity of the sap in some cases fell (in the course of 22 days) to 1.0195 (taking water at 25°C. as 1.000).

We may surmise that if the ocean contained a little more NH_4Cl many organisms might float which do not now do so. Such a change has great biological importance since it profoundly alters the environment (and the distribution) of an organism to bring it from the bottom to the surface of the water. Some organisms such as *Halicystis*⁵ float by excluding certain substances, especially sulfates. It would be exceedingly interesting to know how many mechanisms are employed for flotation and how it can be experimentally produced or suppressed.

Experiments with flotation produced by increasing the specific gravity of the sea water by adding CsCl also gave striking results. When 1 volume of CsCl 0.6 M was added to 9 volumes of sea water the halide content and osmotic pressure remained about the same as

TABLE I.

Specific gravity (at 25°C. taking water at 25°C. as 1.000)		Remarks
Sea water.....	1.02250	Cells sink
9 volumes sea water + 1 volume CsCl 0.6 M.....	1.02740	Cells float
Normal sap.....	1.02250	Cells sink
Sea water containing 0.005 M NH_4Cl	1.02245	Cells sink
Sap of cells which had been 22 days in sea water containing 0.005 M NH_4Cl	1.01950	Cells float

in ordinary sea water; the specific gravity rose from 1.0225 (ordinary sea water) to 1.0274. Cells sink⁶ in ordinary sea water but after the addition of CsCl they float (see Table I). It is evident that they would sink if sufficient CsCl should penetrate but this did not happen although the cells lived for more than a year: the only cells that sank during this time were a few that died.⁷ This experiment recalls that of Loeb⁸ on *Fundulus* but differs in that the external medium was not toxic nor of higher osmotic pressure than the sea water.

⁵ This was formerly called *Valonia ventricosa*: for an account of its flotation see Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924-25, vii, 633.

⁶ The specific gravity of the sap is normally about the same as that of sea water but the weight of the cellulose wall and of the protoplasm causes the cells to sink.

⁷ The cells were kept in finger bowls covered by plates of glass near a north window.

⁸ Loeb, J., *Biochem. Z.*, 1912, xlvii, 127.

The experiment was discontinued after 13 months and the sap was extracted by piercing the cells with a sharp glass capillary (after rinsing the outside for a few seconds with distilled water and drying lightly with filter paper). It was found that the halide concentration of the medium (sea water containing CsCl) had risen, as the result of slow evaporation, from 0.564 M to 0.858 M: that of the sap⁹ had risen to 0.885 M. The specific gravity of the medium had risen from 1.0274 to 1.0473 and that of the sap⁹ to 1.0410.

We are indebted to Professor G. P. Baxter for making a spectroscopic examination of the sap: this showed a very small amount of Cs (probably less than 0.1 per cent); this might have been due to injury of some of the cells, or to contamination of the sap during its extraction. In any case it is evident that little or no Cs penetrated the normal cells during a period of over a year.

A similar experiment was made with RbCl by adding 1 volume of RbCl 0.6 M to 4 volumes of sea water, making the concentration of RbCl 0.12 M. This produced scarcely any change in freezing point depression but raised the specific gravity from 1.0225 to 1.0271. Some of the cells sank after a day and appeared normal but others floated for a few days, after which the experiment was discontinued.

It is therefore evident that there is little or no penetration of CsCl but it seems probable that there is more penetration of RbCl. We know that KCl and NaCl penetrate (either as such or perhaps as KOH and NaOH)¹ since they are found in the vacuole, but our experiments show that such penetration is very slow.

It would seem that K penetrates most rapidly and Cs least rapidly, the probable order being $K > Na > Rb > Cs$. Li also penetrates but its position in the series is doubtful. It may be that the alkalis¹⁰ penetrate the protoplasm as undissociated molecules (e.g. as hydrates)¹¹ whose solubility in the protoplasm may increase in the order given. Höber¹² has recently suggested that the order of penetration is that

⁹ The normal halide content of the sap is about 0.028 M greater than that of the surrounding sea water and its specific gravity not far from that of sea water.

¹⁰ Nothing is said in this connection regarding NH_4^+ since it probably penetrates to a considerable extent as NH_3 .

¹¹ See Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 234.

¹² Höber, R., and Höber, J., *Arch. ges. Physiol.*, 1928, ccxix, 260.

of the ionic radius but this would require that Cs^+ should enter more readily than K^+ .

It should be borne in mind that these ideas cannot be applied generally since in *Halicystis* (formerly called *Valonia ventricosa*¹³) the composition of the sap suggests that Na penetrates more rapidly than K.

It may be of interest to consider the absolute amounts taken up. The best data are those on the absorption of K, Na, and Cl under favorable circumstances. Here, as in other experiments on strong electrolytes, conditions which favor growth favor penetration (as is to be expected since the relation of the salt content of the sap to that of the sea water remains nearly constant). It might be expected that even when no growth occurs cations might penetrate as the result of exchange but our experiments (e.g. attempting to exchange K for Na by increasing the K content of the sea water) indicate that such processes must be very slow. Cells placed in bottles and suspended in the ocean (from July 1 to September 5) increased in weight at the rate of about 1 per cent per day.¹⁴ If we take as an average representative of these cells a prolate spheroid 1.3 cm. \times 0.78 cm. (this measurement does not include the cell wall) we should have for the surface area of each cell $2\pi [b^2 + (ab \div e) \sin^{-1}e]$ where a = one half the major axis ($= 0.65$), b = one half the minor axis ($= 0.39$), and e , the eccentricity, is 0.8 (from the equation $e^2 = (a^2 - b^2) \div a^2$). This gives for the surface 2.8 sq. cm. The volume is $(4 \div 3)\pi ab^2 = 0.41$ cc.

Since the concentration of Cl in the sap is about 0.6 M and the concentration of K¹⁵ is 86.2 per cent of this, 1 liter of sap contains $(0.6) \times (0.862) = 0.5172$ mol of K, and one cell has approximately $(0.41 \div 1000) 0.52 = 0.000213$ mol of K and takes up per day 1 per cent of this or $0.00000213 = 213 \times 10^{-8}$. Since the area of the cell is 2.8 sq. cm. the amount taken up per day per sq. cm. is $(213 \times 10^{-8}) \div 2.8 = 76 \times 10^{-8}$, or $(76 \times 10^{-8}) \div 24 = 3.17 \times 10^{-8}$ mols of K per hour per sq. cm. The intake of Na is this amount multiplied¹⁶ by

¹³ Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924-25, vii, 633.

¹⁴ For measurements of growth see Brooks, M. M., *Am. J. Bot.*, 1925, xii, 617.

¹⁵ This was determined by the perchlorate method.

¹⁶ The molar proportion of Na to K in the sap is $15.1 \div 86.2 = 0.17$.

0.17 or 0.55×10^{-8} mol of Na per hour per sq. cm.: that of Cl is equal to the sum of K and Na or 3.7×10^{-8} .

It would be interesting to compare these figures with those of Northrop¹⁷ for diffusion through a collodion membrane but it would be necessary to know the pressure which drives K and Na into the cell and we are not able to calculate this with certainty. It may be noted that K moves into the *Valonia* cell against the concentration gradient (the concentration of K is about 40 times as great inside the cell as outside) but Na moves with the concentration gradient (the concentration of Na is nearly 6 times as great outside as inside).

Some experiments with anions may be mentioned here. Cells were placed in a mixture of 3 volumes of sea water + 1 volume of NaI 0.6 M where they floated. After 24 days (under conditions favorable for growth) all the cells still floated except those that had died: the iodide in the sap of the living cells was 0.045 M and in the sea water 0.150 M, but when the growth of the cell is taken into consideration it appears that little or no Cl came out of the cell during the absorption of I.

The case seems to be similar with the intake of Br, at least qualitatively. When 3 volumes 0.6 M NaBr are added to 1 volume of sea water so that the cells float, they sink much sooner when conditions are favorable for growth, indicating that Br is taken up during the process of growth.

A recent paper by Höber and Höber¹² states that considerable bromide penetrates in 7 hours. This is more rapid penetration than occurs in our material unless the cells are injured (we determined Br by the method they employed).

We may conclude that in general the penetration of strong electrolytes into *Valonia* is very slow unless the cells are injured.¹⁸ There

¹⁷ Northrop, J. H., *J. Gen. Physiol.*, 1927-28, xi, 233.

¹⁸ In some cases there is visible temporary injury (as evidenced by the disarrangement of chloroplasts when viewed under the microscope) which is followed by recovery: such experiments are rejected. The best criteria of injury are the microscopic appearance, the turgidity, the absence of disturbance to the protoplasm when the cell is rolled between the fingers or bounced lightly on a table, and the presence of sulfate in the sap. In addition cells should be transferred to sea water and kept under observation but this may tell us nothing in regard to temporary injury during the experiment. Cf. Irwin, M., *J. Gen. Physiol.*, 1928-29, xii, 147.

appears to be slow penetration with Li, BrO_3 , IO_3 , and selenite, but other substances show much less penetration, e.g. SCN , ferricyanide, ferrocyanide, formate, salicylate, tungstate, selenate, NO_2 , SO_3 , Sb-oxide, glycerophosphate, and many heavy metals and the alkaline earths.

On the other hand our experiments and those of others show that certain weak electrolytes enter *Valonia* more rapidly, e.g., NH_3 ,¹⁹ H_2S ,²⁰ CO_2 ,²⁰ and others.

We might explain these facts on the ground that the protoplasmic surfaces consist of non-aqueous layers²¹ through which electrolytes pass for the most part in the form of undissociated molecules. We may imagine that ions striking the outer surface of the protoplasm unite to form molecules and as such pass through the non-aqueous surface layer only to dissociate again on reaching an aqueous phase.¹

If ions penetrate as such it may be by exchange of those of the same sign going in opposite directions²² or by the entrance of ion groups (e.g. K^+ and OH^-) formed at the surface by collision of ions of opposite sign which then penetrate together. It would seem that such pene-

¹⁹ For experiments on penetration of NH_3 into *Valonia* see Brooks, M. M., *Pub. Health Rep.*, 1923, xxxviii, 2074. For experiments on *Nitella* see Irwin, M., *J. Gen. Physiol.*, 1925-26, ix, 235. For experiments on other cells see Warburg, O., *Z. physiol. Chem.*, 1910, lx, 305. Harvey, E. N., *Carnegie Institution of Washington, Pub. No. 183*, 1914, 131. Jacobs, M. H., *The Harvey Lectures*, 1926-27, 146.

²⁰ Osterhout, W. J. V., *J. Gen. Physiol.*, 1925, viii, 131. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, ix, 255. For the results of other workers on weak acids see Loeb, J., *Biochem. Z.*, 1909, xv, 254; 1910, xxiii, 95; *Arch. ges. Physiol.*, 1897-98, lxix, 1; 1898, lxxi, 457; Artificial parthenogenesis and fertilization, Chicago, 1913, 143; *J. Gen. Physiol.*, 1922-23, v, 231. Harvey E. N., *Internat. Z. physik.-chem. Biol.*, 1914, i, 463; *Carnegie Institution of Washington, Pub. No. 212*, 1915. Crozier, W. J., *J. Gen. Physiol.*, 1922-23, v, 65, with references to earlier papers. Haas, A. R. C., *J. Biol. Chem.*, 1916, xxvii, 225. Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457; *Biol. Bull.*, 1922, xlii, 14. Brooks, M. M., *Pub. Health Rep.*, 1923, xxxviii, 1449, 1470. Beerman, H., *J. Exp. Zool.*, 1924-25, xli, 33. Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxxviii, 183. Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347.

²¹ Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, xi, 83.

²² Doubtless the cell can produce a sufficient number of ions for exchange, e.g. H^+ and HCO_3^- . Cf. Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxvi, December.

tration must be small in view of the fact that Dr. Blinks finds the electrical resistance of the protoplasm in *Valonia* to be very high. This is so, for example, when the protoplasm is in contact with NH_4Cl although the rise in the pH value of the sap shows that either NH_3 or NH_4OH penetrates rapidly: presumably if NH_4OH penetrates it does so in the form of undissociated molecules.

In the case of strong electrolytes the penetration of undissociated molecules or of ion pairs would probably be much slower than exchange of ions since it could take place only when ions of opposite sign happened to collide at the surface.

SUMMARY.

The entrance of strong electrolytes into *Valonia* is very slow unless the cells are injured. This, together with the very high electrical resistance of the protoplasm, suggests that they may penetrate largely as undissociated molecules formed at the surface of the protoplasm by the collision of ions.

Under favorable circumstances KCl may be absorbed to the extent of 3×10^{-8} mols per hour per sq. cm. of surface together with about 0.17 as much NaCl. Other substances²³ which seem to penetrate to some extent are Li, Rb, Br, BrO_3 , I, IO_3 , and selenite.

Little or no penetration²³ is shown by SCN, ferricyanide, ferrocyanide, formate, salicylate, tungstate, seleniate, NO_2 , SO_3 , Sb, glycerophosphate, and many heavy metals and the alkaline earths.

In sea water whose specific gravity had been increased by CsCl cells of *Valonia* floated for over a year and there was little or no penetration of Cs except as the result of injury.

The penetration of NH_4Cl decreases the specific gravity of the sap and causes the cells to float: under these circumstances they live indefinitely. It is probable that NH_3 or NH_4OH penetrates and is subsequently changed to NH_4Cl . It would seem that if the sea contained a little more ammonia this would be a floating organism.

²³ This statement is based on preliminary experiments only.

THE PERMEABILITY OF DRY COLLODION MEMBRANES. II.

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When freshly made collodion membranes are immersed in water before the complete removal of the organic solvents, the membranes are permeable for all substances of small molecular weight. If the membrane is allowed to dry, however, either before or after placing in water, the permeability to electrolytes is lost and the membranes exhibit in general the same type of permeability as living cells (1-3). They furnish therefore an interesting model of the cell in that the permeability of the "wet" membranes is similar to the dead cell while that of the "dry" membranes is similar to the living cell. The mechanism of the permeability of the "wet" membranes was firmly established by the work of Duclaux and Errera (4) who found that the relative rate of flow of various liquids through these membranes was in proportion to the viscosity of the liquids. There seems no doubt therefore that these membranes consist of a network of capillaries through which the solutions pass. Hitchcock (5) and Bjerrum and Manegold (6) have been able to calculate the size of these pores as of the order of magnitude of 10^{-6} cm. As the percentage of water held in the membrane decreases the size of the pores decreases until with membranes containing 5 per cent or less water the rate of flow of water is too small to be measured and the pore size cannot be determined. Collander has found that semipermeability becomes more marked as the percentage of water decreases and assumes as does Michaelis (7) that this selective action of the dry membranes is due to the fact that the pores become too small to allow the passage of large molecules. The impermeability to electrolytes is accounted for by Michaelis (8) as due to the negative charge on the membrane which prevents the passage of negative ions while secondary differences in the rate of passage of the ions are ascribed to

differences in the degree of hydration of the ions. The potential differences observed by Michaelis are accounted for as diffusion potentials caused by the great differences between the mobilities of the anion and cation in the pores of the membrane. An alternative hypothesis is that the pores disappear in the dry membrane and that substances pass by dissolving in and diffusing through the collodion. The essential difference between the two points of view consists in that from the point of view of pores, molecules in solution are surrounded by an atmosphere of the solvent molecules and really move relative to them, the membrane simply serving to alter the area and length of the column of solvent through which the solute can diffuse. The permeability is therefore determined by the diffusion coefficient in the solvent and by the effective diffusion area of the membrane.

From the point of view of solution the molecules are considered to diffuse through the material of the membrane and the solvent in contact with the membrane affects the permeability only indirectly, in that the concentration of the solute in the membrane depends upon the partition coefficient of the substance between the membrane and the solvent. In the case of diffusion of substances in solution the results can be predicted at least qualitatively by either point of view, although there is evidence in favor of the solution idea. The simplest case appears to be that of the passage of gases; and in this case, as was pointed out in a preliminary paper (3), the results are more in accord with the idea of solution. It may be mentioned that there are undoubtedly cases where passage takes place through pores, as in the wet collodion membrane or in unglazed porcelain, while there are also cases such as the passage of solutes through a layer of ether or of gases through rubber (9) or metals where the process is one of solution.

Experimental Procedure.

Preparation of Membranes.—5 cc. of Merck's u. s. p. collodion was placed in a 1.5×15 cm. test-tube and rotated mechanically in a horizontal position for 20 minutes. The tubes were then allowed to dry for 3 days. They were then filled with water and the membranes removed, drained and dried for 3 days in a desiccator over H_2SO_4 . The membranes could be made thicker or thinner by using more or less collodion. Thinner membranes frequently have imperfections and the thicker ones render the experiments slow.

Attachment of the Membrane.—The glass tube to which the membrane attached was just large enough so that the membrane could be drawn smoothly without wrinkling. The tube was first covered with a thin vacuum stop-cock grease and the membrane slipped on. It was then firmly in place with rubber bands.

Determination of the Rate of Passage of Gases.—The determination of permeability for gases was made in the apparatus shown in Fig. 1. The inner and glass tube were completely filled with gas by running the gas first through the membrane and then into the outside tube, by appropriate manipulations of the stop-cocks. The volume of gas passing through was read from the movement of the

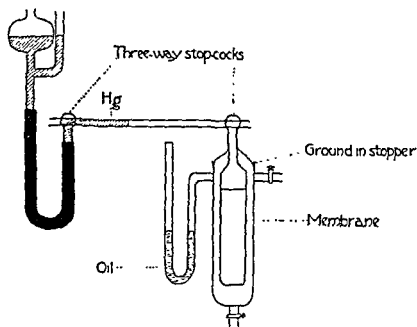


FIG. 1. Apparatus for measuring rate of passage of gases through membranes.

the mercury meniscus and checked by the change in the oil manometer. Since the total volume of the apparatus is large compared to the volume of gas passing through, the oil manometer is very sensitive to changes in barometric pressure and temperature. The entire apparatus was therefore immersed in a constant temperature water bath. Even under these conditions, unless precautions are taken against leaks, etc., the change in the outside manometer did not agree quantitatively with that of the inside one.

Determination of the Solubility of Gases.—The volume of gas taken up by iodine was determined in an apparatus essentially the same as that described by Lefebvre (10). The apparatus containing the collodion was evacuated and then subjected to a millimeter of pressure for several hours before use.

Determination of the Permeability to Solutes.—A glass tube held in a clamp was tied in the end of the membrane and the membrane contained

pended in a test-tube of water. The quantity of solute outside was determined by titration with alkali in the case of acids or by AgNO_3 in the case of chlorides. Phenol was determined by titration with iodine.

Determination of the Solubility of Acetic Acid.—Several grams of collodion membrane were placed in a measured volume of the solution and allowed to remain until no further change in concentration of the solution was noted. This required from a day to several weeks depending on the concentration of the solution and the thickness of the membrane. The collodion was then removed from the solution, rinsed with water and placed in a small volume of water. It was placed in fresh water every day until no further solute was found in the water. The wash waters were then combined and the total amount of solute given off from the collodion determined. This total amount taken up by the collodion was also determined by difference, from the analysis of the original solution before and after the collodion had been placed in it. The figures obtained agreed with each other, showing that the process is reversible. This is not true of NH_3 which forms an irreversible compound after long standing.

Effect of the Size and Thickness of the Membrane and the Time of the Experiment.

From either the point of view of diffusion through pores or of solution and diffusion through the membrane, it would be expected that the general form of the equation governing the process would be that of Fick's diffusion law, which states that the quantity of material passing in unit time is proportional to the area and to the concentration difference across the membrane. The significance of the terms of the equation is different, however, depending on which assumption is used.

Assumption I. Diffusion Takes Place through Pores.

If the experiment is arranged so that there is a "steady state," that is, so that the concentration difference across the membrane is constant, the concentration gradient across the (homogeneous) membrane is linear and the equation may be written

$$Q = \frac{P t A (C_1 - C_2)}{h}$$

(1)

or

$$P = \frac{Q h}{t A (C_1 - C_2)}$$

in which Q is the quantity of substance that passes through area A , of thickness h , in time t when the concentration difference in the solution

or gas on the opposite sides of the membrane is $(C_1 - C_2)$. P may be called the permeability constant, since it is the amount of material that will pass through unit area and thickness of membrane in unit time under unit difference of concentration (or pressure). This relation was found to hold both with gases and with substances in solution. The partial pressure of the gas was used. The amount of substance passing at the beginning of the experiment will evidently be smaller than that at the steady state since some of the substance remains in the membrane, so the rate increases slowly at first and then remains constant. The experiments were continued until this constant rate was reached. This required an hour or so for the gases and several days for some of the substances in solution.

Dimensions of P .—It will be noted that if the area and thickness are expressed in the same units and the pressure or concentration in terms of quantity per unit volume, P then has the dimensions: *area over time*. Since the mole fraction, which is the significant figure, of the molecular species present in small amount (or the pressure of a gas), is nearly proportional to the mass per unit volume, the concentrations of gases and solutes in low concentration may be expressed as quantity per unit of volume. In these experiments the results are expressed as cm.^2 per day. The following is an example of the calculation.

$$A = \text{area membrane} = 72 \text{ cm.}^2$$

$$h = \text{thickness membrane} = 26 \times 10^{-4} \text{ cm. (calculated from area and weight)}$$

$$t = 2 \text{ days}$$

$$Q = 2.2 \times 10^{-6} \text{ mole}$$

$$C_1 = \frac{10 \times 10^{-6} \text{ mole}}{\text{cm.}^3}$$

$$C_2 = 0$$

$$P = \frac{2.2 \times 10^{-6} \text{ mole} \times 26 \times 10^{-4} \text{ cm.}}{72 \text{ cm.}^2 \times \frac{10 \times 10^{-6} \text{ mole}}{\text{cm.}^3} \times 2 \text{ days}} \quad (2)$$

$$= 0.040 \text{ cm.}^3 \text{ per day.}$$

Evidently it makes no difference what units are used to express the quantity and concentration as long as the units are the same in both cases. In the case of a gas, grams or cc. per cm.^3 at standard pressure and temperature, can be used. In a solution, however, the mole,

fraction of the *solvent*, *i. e.* the molecular species present in excess, is not proportional to its volume concentration, and in this case the dimensions of P cannot be reduced to area over time but must be expressed as quantity per unit membrane size per unit of time and unit of osmotic or vapor pressure, or whatever property of the solvent is assumed to be the determining factor.

From the point of view of pores the value of P is the diffusion coefficient of the substance in water or in whatever substance is supposed to fill the pores of the membrane. It differs from that determined in water without a membrane because it has been expressed per unit area of membrane whereas the area and length of the pores only should have been used. It follows from this point of view that the permeability is a function of the diffusion coefficient of the substance in the solvent and of the effective pore area and length, so that the membrane affects the value only by changing the effective diffusion area. Evidently then the rate of diffusion of the same substance should be different depending on the solvent. A gas for instance should diffuse much more rapidly through a membrane in the gas, in which case the pores are filled with the gas, than through the same membrane immersed in water since the pores would now be filled with water and the rate of diffusion of gases in gases is some 10,000 times as great as the rate of diffusion of gases in water. As will be seen, this is not the case. It would also be predicted that the relative rates of penetration should be in the same order as the diffusion coefficients in water, but the differences should be magnified since the percentage of the total number of pores through which small molecules can pass is greater than that for large molecules. In general this is true, but there are marked exceptions since electrolytes do not pass with appreciable velocity whereas some large molecules, as phenol, pass rapidly. In the case of large molecules having a higher rate of penetration than small ones it is necessary to assume that the diffusion law does not hold in small capillaries or that the substance becomes concentrated in the capillaries.

Assumption II. The Substances Dissolve in and Diffuse through the Material of the Membrane.

The equation for the rate of penetration is the same as before but from this point of view the terms referring to the dimensions of the

membrane are correct but the concentration terms must now refer to the concentration in the two sides of the membrane instead of the concentration in the solution.* If S is the partition coefficient of the substance between the solvent and collodion, *i. e.*

$$S = \frac{\text{Concentration in collodion}}{\text{Concentration in solvent}} \quad (3)$$

then the concentration in the collodion is SC and equation (1) may be written

$$Q = \frac{t D A S (C_1 - C_2)^\dagger}{h} \quad (4)$$

or

$$D = \frac{Q h}{t A S (C_1 - C_2)}$$

$$= \frac{P}{S}$$

D has now the same dimensions as P in the previous case but is of different significance, since it is the diffusion coefficient of the substance in the collodion (more strictly, in a saturated solution of whatever substances are present in the collodion). From this point of view differences in Q , the quantity passing through, are due to differences in the partition coefficient of the substance between the solvent and collo-

* It is assumed, in accord with the work of Noyes and Whitney and others on solution of solids, that equilibrium exists at the interface and that the time element consists in the diffusion. This is a reasonable assumption since the actual interface is of molecular dimensions and the activity of a substance at any point in a solution would differ very slightly from that at another point distant by a few molecular diameters. Unless there were some special block at the interface the activity on the two sides would therefore be expected to be practically identical, *i. e.*, there would always be equilibrium at the interface.

† If the solutions on the two sides are different the partition coefficients will be different and the general equation is

$$Q = \frac{t D A (S_1 C_1 - S_2 C_2)}{h}$$

This is the case in the experiments reported by Irwin (17).

dion and to the diffusion coefficient of the substance in collodion. This value, D , should therefore depend only on the substance and should be independent of the material surrounding the membrane. Gases therefore should give the same value for D when the membrane is surrounded by gas as when it is in water, and this is the case. The

	<i>Peri</i>			
Substance.....	H ₂	H ₂	NH ₃	H ₂ O
System measured.....	$\frac{H_2}{H_2}$	$\frac{H_2}{H_2O}$	$\frac{NH_4OH}{H_2O}$	$\frac{H_2O}{Sugar}$
Moles per liter or pressure at.....	0.05-1.0	1.0	0.10	0
P = permeability = $\frac{cm.^2}{day} \times 10^4$	7.5	7.3	1.2	
$S = \frac{gm. \text{ per cc. collodion}}{gm. \text{ per cc. water or gas}}$10	.10	2.0	.07
$D = \text{Diffusion coefficient in collodion}$ = $\frac{P}{S} = \frac{cm.^2}{day} \times 10^4$	75	73	0.6	30-100
Molecular weight.....	2	2	17	18
Molecular radius $\times 10^8$ cm.....	1.4	1.4		1.44

values for the permeability should have no general relation to the molecular weight or size except in so far as the partition coefficient between water and collodion varies with these quantities. The values of D should decrease as the size of the molecule increases, although there is no known relation between the diffusion coefficient and the molecular diameter or weight except when the molecule of solute is large compared to that of the solvent. In that case the diffusion coefficient is

inversely proportional to the diameter of the molecule or to the cube root of the molecular weight (11). Empirically it is known that in water the diffusion coefficient of molecules of the same order of size as the water molecule is inversely proportional to the square root of the molecular weight. In the present case the molecules of the solvent,

E I.

ion Membranes.

HCl gas	HCl	CO ₂	CO ₂	CO ₂	HCOOH	CH ₃ COOH	CH ₃ NH ₂ COOH	C ₂ H ₅ OH	CH ₃ ClCOOH	CHCl ₃ COOH	HgCl ₂
$\frac{\text{HCl}}{\text{HCl}}$	$\frac{\text{HCl} - \text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{CO}_2}{\text{CO}_2}$	$\frac{\text{CO}_2 - \text{H}_2\text{O}}{\text{CO}_2 \text{ H}_2\text{O}}$	$\frac{\text{CO}_2}{\text{O}_2}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$	$\frac{\text{H}_2\text{O}}{\text{H}_2\text{O}}$
.05-.10	0.1	0.05-1	1	1	0.1	0.01-1.0	0.10	0.2	0.1	0.1	0.1
240	0	6.7	8.0	7.0	.16	.038	0	.15	.025	.0033	.003
>100	0	4.6	4.6	4.6	.75	1.0	0	2.8	.36	.11	.16
2.4?	?	1.4	1.7	1.5	.21	.038	?	.054	.07	.03	.018
36		44			46	60	75	90	94	128	270
1.59		1.61									

collodion, are much larger than those of the solute, and it might be expected that the rate of diffusion would vary as some higher power of the molecular weight.

Experimental Results.

A summary of the results is given in Table I, in which the substances have been arranged in order of their molecular weight. The permea-

bility decreases in an irregular way as the molecular weight increases but there are marked exceptions. HCl gas for instance passes many times more rapidly than hydrogen; and phenol, although of large molecular weight, passes many times more rapidly than acetic acid; while amino acetic does not pass with measurable velocity. The clearest results are those for the gases, since the theory of the passage of gases through fine openings is well worked out and there is less chance for secondary complications. It is known that under all conditions the relative volume of gas passing through a small opening is nearly inversely proportional to the square root of the density of the gas, and that this relation holds even when the capillary is of the order of magnitude of the mean free path of the gas molecule (12). Hydrogen should therefore pass the most rapidly and CO₂ the most slowly. CO₂ and H, however, pass at the same rate while O and N pass much more slowly and HCl goes very much more rapidly. On the other hand, it can hardly be assumed that CO₂ and HCl can pass through pores that will not admit H and the other gases, since the relative diameters of the gas molecules are known and do not differ much, CO₂ being the largest. (There are several independent methods of determining the molecular radius, which lead to different results. The relative order of size, however, is the same for all methods; *cf.* Loeb (12).) On the other hand, when the results are expressed as coefficients of diffusion in collodion, the results are correctly predicted qualitatively for all the substances measured, without any supplementary hypotheses as to electrical effects, etc. Those substances that do not pass the membrane do not dissolve in it, and those like phenol, which are exceptions from the point of view of pores, are found to be soluble. The most striking examples of the effect of solubility, such as NH₃ gas, H₂S gas, and to a certain extent HCl and the collodion solvents of higher molecular weight, cannot be tested from the point of view of solubility since they either destroy the membrane or combine with it irreversibly so that the solubility coefficient cannot be determined. Collodion was found to take up large quantities of NH₃ and H₂S, but the membrane changes color and becomes brittle while only a small part of the gas can be removed. Evidently a chemical reaction takes place subsequent to solution.

TABLE II.
Effect of Water on Passage of H₂ through Collodion.

$$\text{Membrane factor} = \frac{h}{A} = 2 \times 10^{-5}.$$

	Dried over H ₂ SO ₄ Dry air 1 atmosphere	Saturated with H ₂ O vapor Air saturated with H ₂ O 1.1 atmosphere	Immersed in H ₂ O H ₂ O, air bubbled through H ₂ 1.1 atmosphere
Membrane.....			
Outside.....			
Inside.....			
Partial pressure H — atmosphere.....	1.1	1.1	1.1
Cc. H ₂ per hr.....	2.2	2.3	2.05
$P = \frac{\text{cm.}^2}{\text{day}} \times 10^4$	9.6	10.0	9.0
Order of running experiment.....	1	2	3

The diffusion coefficients of the substances in collodion increase as the molecular weight decreases, which is a reasonable result. The increase, however, is much more rapid than would be expected from the relative rates of diffusion in water, and although no theoretical relation is known between the diffusion coefficient and the molecular weight when the solute molecule is smaller than that of the solvent it appears hardly probable that the diffusion coefficient should increase as rapidly as the figures show. It will be noted, however, that those substances having a molecular weight of 60 and above give diffusion coefficients of about the relative order expected from the molecular weight, while those for O, CO₂ and N are also consistent with each other, as are the values for water and hydrogen. It might be supposed from these results that there are some very small pores and that while the large molecules pass by solution and diffusion the small molecules can also pass through pores. This assumption, however, appears to be ruled out by the fact that H and CO₂ pass at the same rate whether the membrane is immersed in water or in the dry gas.

The result of such an experiment with hydrogen and either a dry membrane or membrane immersed in water is shown in Table II. The rate of passage of the hydrogen is the same within the error of the readings. This is difficult to account for if it is assumed that the hydrogen passes through pores, since, when the membrane is surrounded by gas, the pores must be filled with gas molecules, while when the membrane is in water the gas must diffuse through water, and it is known that the rate of diffusion of gases in water is about 1/10,000 the rate of diffusion of gases in gases. It can hardly be supposed that the pores allow the hydrogen to enter but not the water since the hydrogen molecule is very slightly smaller than the water. Neither can it be assumed that the pressure forced the water out of the pores since the pressure necessary to force water out of such small pores would be many hundreds of atmospheres. From the point of view of solution and diffusion the result is exactly what is expected since the difference in partial pressure of the hydrogen on the two sides of the membrane is 1.1 atmospheres in both cases and the rate of diffusion through the collodion should therefore be the same.

Table III gives the result of a similar experiment with CO₂. In this case the amount of gas passing the membrane was determined by

titration in the outside solution instead of by loss of volume on the inside, so that there is no doubt that the measurement represents the

TABLE III.
Diffusion CO₂, Membrane in H₂O.

	Outside			H ₂ O sat. + CO ₂		
	Inside	H ₂ O + m/100 NaOH.		Time required to bring to pH 8.0 determined		
Membrane No.	2			4		
F × 10 ⁵	2.8			2.8	CO ₂ /CO ₂	
Cc. m/100 NaOH	1	1	1	1		
≈ cc. CO ₂	.224			.224	.23	
Δt, hrs. → pH 8.0	.17	.18	.18	.26	.21	
Average	.17			.22		
Cc. CO ₂ /day/at	31			24		
P = cm. ³ /day × 10 ⁴	8.6			7.4	6.7	

CO₂ diffusion coefficient in H₂O = 1.2 cm.²/day

in O₂ = .18 cm.²/sec. = 1.6 × 10⁴ cm.²/day

TABLE IV.

Effect of Water on Permeability of Membrane with Small Holes.

Membrane filled with air under 3 cm. Hg pressure.

8 punctures made in membrane with fine glass needle and rate measured in air and in water.

	Membrane in air	Membrane in H ₂ O
Cc. per hr.....	4000	0 3

TABLE V.

Effect of Area and Method of Preparing Membrane on Quantity of CO₂ Absorbed.

Method of preparing membrane.....	Poured into water	Membrane made in test-tubes	Membrane made on mercury	
Area membrane per gm. collodion. cm ²	?	1100	120	50
Cc. CO ₂ per cc. collodion 760 mm. pressure.....	4.8	5.1	4.7	4.7
	4.7			
	4.3			

amount of gas passing through the membrane. Since the radius of the CO₂ molecule is known to be as large or larger than that of water it cannot be assumed that the water was unable to enter the pores.

The experiment was controlled by measuring the rate of diffusion of air through a membrane which had been punctured with a fine glass needle. The result of this experiment is shown in Table IV. The gas now passes more than 10,000 times as fast when the membrane is in air as when it is in water.

The foregoing experiments agree qualitatively at least with the assumption that the passage of substances is governed by solution rather than by diffusion through pores. It is possible, however, to obtain confirmatory evidence from several different experiments.

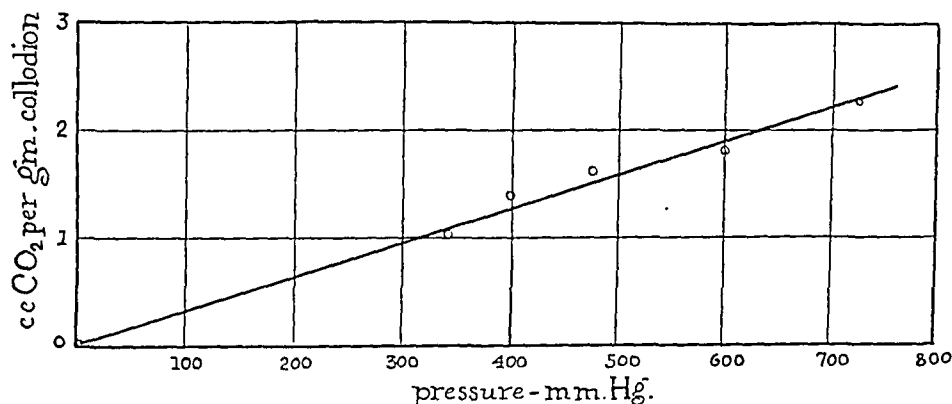


FIG. 2. Solubility of CO₂ in collodion at different pressures.

Effect of Pressure or Concentration and Form of Membrane on the Amount of Substance Dissolved in Collodion.

If the substances form a solution in the collodion it would be expected that the quantity dissolved would be proportional to the concentration or pressure of the substance and independent of the surface of the collodion. Table V shows that the amount of CO₂ absorbed by collodion is independent of the area of the collodion and is nearly the same whether the collodion is prepared by pouring into water or made in the form of a dry membrane. The solubility is a little less in the water sample and it was noted also that some samples of collodion showed a slightly different value for the solubility. Collodion is known to be a mixture and it is possible that the solubility in the various constituents is different, so that different samples would be

expected to vary slightly. If this were the case it might account for the rapid decrease of the diffusion coefficient with increasing molecular weight. But it must be assumed that the membrane is heterogeneous in the plane *perpendicular* to the direction of diffusion.

The amount of CO_2 dissolved by collodion at different pressures is shown in Fig. 2. The quantity is proportional to the pressure up to pressures of 760 mm. Hg.

TABLE VI.
Solubility of Acetic Acid and Phenol in Collodion.

Concentration in H_2O	Concentration in collodion		Partition coefficient = $\frac{\text{Concentration in collodion}}{\text{Concentration in } \text{H}_2\text{O}}$	
Acetic acid or phenol	Acetic acid	Phenol	Acetic acid	Phenol
mm per cc.	mm per cc.	mm per cc.		
0.01	0.01		1.0	
.025		0.071		2.8
.05		(.20)		(4.0)
.10	.099	.28	1.0	2.8
.20		.57		2.8
1.0	.87		.87	
5.0	3.05		.61	

TABLE VII.
Solubility of Acetic Acid in Collodion from Acetic Acid-Sodium Acetate Mixtures.

Cc. N/10 HA.....	10	8	5	2	0
Cc. N/10 NaA.....	0	2	5	8	10
Moles HA per cc. collodion.....	.88	.91	1.0	1.0	
Moles HA per cc. H_2O					

The solubility of acetic acid and phenol in solutions of different concentrations in collodion is shown in Table VI. The solubility of acetic acid is proportional to the concentration up to about molar, but above that the concentration of the acetic acid in the collodion increases more slowly than in the solution. The collodion begins to soften at about this concentration of acetic acid and can probably no longer be considered as pure collodion.

Since acetic acid is soluble in collodion while sodium acetate is not, it may be expected that the amount of acetic acid dissolved from a mixture of acetic acid and sodium acetate having the same total acetate concentration would be proportional to the amount of undissociated acetic acid in the mixture. Table VII shows that this is the case.

Independent Solubility of Water and Acetic Acid, etc.

According to the theory of ideal dilute solutions the quantity of substance dissolved should be the same irrespective of the presence of

TABLE VIII.
Weight and Composition of Solution Taken Up.

Solution	Acetic acid		HgCl ₂	Phenol
	1 M	5 M	.10 M	.2 M
Weight collodion dry gm.422	.422	.434	.287
“ “ + H ₂ O.440	.440	.451	.298
“ “ + solute.452	.480	.4542	.306
Gm. solute in collodion.012	.040	.0032	.008
≈ moles solute × 10 ⁵	20	66	1.2	8.5
Moles per gm. collodion × 10 ⁵	47.5	156	2.7	28
Found by direct titration × 10 ⁵	48	170	2.5	26
Moles per gm. collodion assuming solution of same concentration taken up by collodion.	7	14	.46	1.3

Assume adsorption and that each mole HA displaces 1 mole H₂O.

Then total weight per gm. = 170 moles × 10⁻⁵ HA + (240-170) × 10⁻⁵ moles H₂O

= .1 gm. HA + .012 gm. H₂O

= .112 gm. total

Found .137

other solutes. The amount of water and of various solutes taken up by dry collodion is shown in Table VIII. The results agree with the assumption that the water and solute dissolve independently and cannot be accounted for by supposing that the membrane simply absorbs solution of the same concentration as the bulk of the solution. If it is assumed that the acid is adsorbed in the pores and that each mole of acid displaces one mole of water, the calculation also fails to agree with the experiment. It would be necessary from this point of view

to suppose that each mole of acid displaced much less than one mole of water, or else that the volume of the collodion increased.

Effect of Sodium Chloride on the Permeability to Acetic Acid.

If the acid diffuses through pores in the membrane it might be expected that the presence of concentrated salt solution on the opposite side of the membrane would increase the amount of acid passing through, since the salt forces water to pass through the membrane owing to osmotic pressure and some acid would be carried with the water. On the other hand, the presence of salt in solution with the acid would cause a stream of water to flow toward the acid and might be expected to decrease the amount passing through the pores. There are, however, possible secondary electrical effects or blocks due to partial closing of the pores by salt molecules, so that it is difficult to predict exactly the result of the experiment from this point of view. From the point of view of solubility the presence of the salt on the outside of the membrane should have a negligible effect since the concentration of acid is here practically 0. The presence of salt on the inside should increase the amount of acid passing through, since strong salt solutions increase the activity (vapor pressure) of the acid and should therefore increase the partition coefficient, and hence the permeability. The results of the experiment given in Table IX show that this latter prediction is qualitatively fulfilled but that quantitatively the increased solubility of the acid in the collodion due to the salt is not sufficient to account for the increase in permeability.

Temperature Coefficient.

If the substance diffuses through pores in the membrane the temperature coefficient of permeability should be that found in ordinary diffusion, unless it be further assumed that the pore area of the membrane changes with temperature. If passage takes place by solution almost any temperature coefficient might be expected since the temperature coefficients of solubility are irregular and may even be negative. The temperature coefficient of the *diffusion coefficient* in collodion should be small, however, unless some change occurs in the viscosity of the collodion. Table X gives the results of an experiment in which the rate of penetration of acetic acid was determined at 5°, 25° and 35°C. The

TABLE IX.
Effect of NaCl on Permeability of Acetic Acid.

	m/10 HA H ₂ O	m/10 HA 5 M NaCl	m/10 HA + 5 M NaCl H ₂ O	m/10 HA 5 M NaCl
Inside.....				
Outside.....				
Permeability HA				
cm. ² /day $\times 10^4$028	.029	.042	.027
Diffusion HA in collodion				
cm. ² /day $\times 10^4$028	.028	.035	.027
Partition coefficient HA				
in collodion from.....	5 M NaCl solution = 1.2 .1 M HA			

coefficient is about 2 for permeability over the whole range, and 1.6 for the diffusion coefficient from 5 to 25, but is the same as that for permeability from 25 to 35.

Permeability to Water.

Membranes made by placing in water before evaporation of the organic solvents may contain more than 5 times as much water as collodion but this water is not in equilibrium since it cannot be replaced once it has been removed without subjecting the membrane to some organic solvent. There is good reason to suppose that this water is held in the pores of the membrane and in fact that the pores are kept

TABLE X.
Permeability of 0.10 Molar Acetic Acid at Various Temperatures.

Temperature	5°		25°		35°
$P = \text{cm}^2/\text{day} \times 10^4$	0.0086		0.038		0.085
Q_{10}		2.1		2.2	
$S = \frac{\text{Acid per cc. collodion}}{\text{Acid per cc. H}_2\text{O}}$53		.90		.94
$D \times 10^4 = \frac{P}{S} =$016		.042		.090
Q_{10}		1.6		2.1	

open by the surface tension effects of this water, as Zsigmondy (13) has suggested.

If such membranes are suspended over mixtures of sulfuric acid and water, or even over pure water, they lose weight and finally come to an equilibrium value with respect to water content. This small amount of water is in equilibrium with the membrane and may be removed and replaced repeatedly. The loss of water over pure water is presumably due to the pressure in the collodion network which causes the water to be forced out in droplets. These droplets have a convex surface and hence a higher vapor pressure than water in bulk. This is a slow process, however, and the loss of weight continues for months. The water content would presumably reach the value obtained by placing dry membranes in water.

it is held, since it is known that the vapor pressure of a liquid which wets a capillary decreases as the radius of the capillary decreases. From this point of view it is possible then to calculate the radius of these residual pores. The result of this calculation is shown in Table XI. It is evident that the calculated size of the pores is too large, since more than half the water is found to be in pores greater than 1×10^{-7} cm. radius or nearly 100 times the cross-sectional area of the molecules of gas which, from the permeability experiments with gases, are unable to pass through them. Also there is no reason to expect, from this point of view, that the amount of water in the pores should be proportional

TABLE XI.

Radius of Pores from Vapor Pressure (14).

$$\ln \frac{P_0}{P} = \frac{2 \sigma M}{RT \delta r}$$

σ = surface tension = 72 ergs/cm.²

M = molecular weight = 18 gm. mole⁻¹

δ = density liquid = 1 gm./cm.³

RT = 2470 $\times 10^7$ ergs mole⁻¹

r = radius capillary cm.

P_0 = vapor pressure liquid in bulk

P = " " " " capillary

$P_0 = 24$

$P =$ 1.2 3 8 15 20

$r \times 10^8$, cm. 3.5 5.0 9.5 22 56

Water in

membrane

as per cent

of maximum 6 10 33 64 90

to the vapor pressure of the water. The calculation, however, includes the doubtful assumption that the angle of contact of the water and collodion is 0. There is also some question as to whether the equation can be applied to such small pores (18).

The permeability of the membranes to water may be determined by filling the membranes with salt or sugar solutions, immersing them in water and determining the increase in weight. Assuming that the water content of the membrane is proportional to the vapor pressure of the solution, the rate of diffusion in the collodion may be calculated. The result of such a calculation is given in Table XII. The vapor

pressure of the solution was calculated from the freezing point depressions in the case of the salt (neglecting the effect of temperature) and assumed proportional to the mole fraction of water in the case of the sugar solutions. It may be noted that the results with the salts although consistent for the various concentrations of the same salt are lower with CaCl_2 than with KCl and sugar. This is the reverse of the result obtained by Lucke and McCutcheon (15) with sea urchin eggs.

If the water is assumed to flow through pores then the diameter of the pores may also be calculated from the above data, as was done by Hitchcock (5) and by Bjerrum and Manegold (6).

This calculation is shown in Table XIII. The size obtained in this way is of the order of magnitude of 10^{-9} , or about 100 times smaller

TABLE XII.

Permeability of Membrane to Water When Filled with Various Solutions.

Solution	Sugar		KCl		CaCl ₂		
	1.0 M	0.50 M	1.0 M	0.5 M	1.0 M	0.5 M	0.25 M
Cc./day/unit membrane $\times 10^5$	1.25	.53	1.2	.60	1.45	.74	.27
Gm. H ₂ O per cc. collodion water side—gm. H ₂ O per cc. collodion solution side.....	.00125	.00062	.00218	.0011	.0033	.00175	.00087
$D = \text{cm.}^2/\text{day} \times 10^4$	100	85	55	55	44	42	31

than from the vapor pressure measurements. It also disagrees with the results of the gas measurements, since the radius is now smaller than the smallest molecular radius. There is again, however, some uncertainty as to the application of the equation to such small pores.

Electroendosmosis.

It might be supposed that the presence of pores could be tested for by electroendosmosis experiments. Since the effect of an electric potential is essentially the same as hydrostatic pressure, it should cause water to pass by diffusion also, and the experiment is therefore inconclusive.

Permeability to Electrolytes.

It has been found by Michaelis and his coworkers that dry collodion membranes act as reversible electrodes for cations but not for anions. That is, in low concentrations of salts a potential approaching the theoretical value of 55 millivolts is obtained when the membrane is placed between 0.10 and 0.01 molar KCl or other univalent neutral salt. These results have been accounted for by Michaelis by the

TABLE XIII.
Calculation of Pore Size and Number from Permeability of H₂O.

Solution	Sugar		KCl		CaCl ₂		
	1.0 M	0.5 M	1.0 M	0.5 M	1.0 M	0.5 M	0.25 M
Cc. per day per cm. ² per cm. thick membrane $\times 10^6$	1.2	.53	1.17	.61	1.45	.74	.27
Osmotic pressure atmospheres.....	22.4	11.2	39.5	19.5	60.5	31.4	15.7
Cc. per day per atmosphere per unit membrane $\times 10^5$056	.048	.029	.031	.024	.023	.017
Radius pore $\times 10^9$ cm.....	2.8	2.6	2.0	2.1	1.8	1.8	1.5
No. of pores per cm. ² $\times 10^{-18}$	1.8	2.0	3.4	3.2	4.2	4.3	5.9

r = radius in cm.

l = thickness collodion in cm. = 1

η = viscosity of water = .01

Q = cc. water per sec. per dyne

m = gm. collodion per cc. = 1.65

$w = \frac{\text{Wet weight}}{\text{Dry weight}} = 1.04$

$n = \frac{m^2(w-1)}{8\pi Q l^2 \eta}$ n = no. of pores per cm.²

assumption that the anions are prevented from entering the pores due to an electrical block. The cations can enter the pores but are held back by the attraction of the anions. When a salt solution is present on both sides of the membrane, therefore, the cations can pass through but the anions cannot, so that in the pores the mobility of the cations is much greater than that of the anions. Under these conditions the diffusion potential in the pores should be the same as that expected from a reversible electrode, and this is the case. It would be expected from this point of view that an exchange of cations should take place

when a salt solution is present on both sides of the membrane. Michaelis (16) has found this to be true, but the amount of cations passing through the membrane is exceedingly small. The membranes used in the present experiments gave a concentration potential when placed between 0.10 and 0.01 M KCl of 30 to 40 millivolts. They were impermeable to all electrolytes tried except HgCl_2 . This salt is more soluble in alcohol than in water and has a measureable solubility in collodion, as might be expected. As noted in Table I it is fairly permeable. About one-third of the membranes tested showed measurable permeability to other electrolytes but the results were irregular and the majority showed no permeability even after a month. It seems necessary to assume that in such experiments the minimum figure is the correct one. The experiments in which the membrane was placed between two salt solutions were also irregular. In the majority of cases no exchange of cations could be noted while in those cases where an exchange occurred there was also some passage of anions. It is possible that this difference from Michaelis' results is due to the different collodion and method of making the membranes. In the case of sodium or potassium acetate separated from HCl, however, there is regularly a passage of the salt through the membrane while no passage occurs when pure water is on the other side of the membrane. The result of such an experiment is shown in Table XIV. It will be noted that both ions of the salt pass through. This result would be expected, however, if the cations could exchange as suggested by Michaelis, since in this case the acetic acid formed can penetrate. If potassium ions passed through, therefore, in exchange with hydrogen ions, acetic acid would be formed in the acetate solution and since the acetic acid could penetrate it would in turn diffuse through and this would continue until the concentration (activity) of the undissociated acetic acid became equal on the two sides of the membrane. The net result would be that both potassium and acetate ions pass through the membrane. From the point of view of solubility these results can be accounted for by assuming that the collodion contains a small amount of an organic acid in solution whose salts are also soluble in collodion. (16). The membrane would therefore act as a reversible electrode for cations as soon as an amount of cation equivalent to the organic acid was taken up since the concentration of the cation in the membrane

would now be constant, a necessary and sufficient condition for a reversible electrode. The low potentials found by Michaelis between concentrated salt solutions would on this basis be due to a very slight independent solubility of the salt itself in the membrane. This explanation has an advantage in that it predicts that the exchange of cations would be very small. As shown in Table XIV this is the case, the penetration of potassium or acetate ion being about 1/10 that for acetic acid. If the impermeability to these ions were due to an electrical block which prevented them from entering the pores, it would be expected when the block was removed by arranging the experiment so that an exchange of ions could take place that the rate of passage of the ions would be of the same order of magnitude as that of molecules of the same size.

TABLE XIV.
Permeability of Potassium Acetate, 30 Days.

Inside.....	0.10 M. KA		0.10 M. KA	
Outside.....	.1 M. HCl		H ₂ O	
Moles Cl ⁻ inside × 10 ⁵ ..	2.0	0		
Moles outside × 10 ⁵	K 6.6	4.0	Trace	Trace
	Acetate 6.0	5.0	"	"
Membrane factor.	2 × 10 ⁻⁵	2 × 10 ⁻⁵	2 × 10 ⁻⁵	2 × 10 ⁻⁵
Permeability × 10 ⁷	Cl 1.0	0		
	K 3.3	2.0	0	0
	A 3.0	2.5	0	0

It is also possible that the membranes contain a few small pores having the properties assumed by Michaelis. The potential developed is independent of the number of pores so that the electrical properties of the membranes may be determined by these occasional and perhaps accidental openings, whereas the actual permeability is determined by solubility and diffusion since if the total pore area is minute the amount of substance passing through them would be negligible.

An essential difference between the two mechanisms exists in the prediction of the results with electrolytes. If the permeability depends on pores and the electrolytes are prevented from passing by an electrical block, then no strong electrolyte can pass. If, however, the permeability depends on solution in the collodion it is quite possible that some electrolytes would have this property and the membrane

would therefore be permeable to them. Mercuric chloride, however, can hardly be cited as an example of this prediction since it is only slightly ionized.

SUMMARY.

The rate of penetration and the solubility of H, O, N, NH_3 , H_2O , HCl gas, CO_2 , formic, acetic, chloracetic, dichloracetic acid, glycerol, phenol and mercury bichloride in dry collodion membranes have been measured.

The rate of penetration of H and CO_2 is the same whether the membrane and gas are dry or whether the membrane is immersed in water.

The solubility of CO_2 , acetic acid, phenol and water in collodion is completely reversible and is proportional to the concentration (or vapor pressure) in low concentrations and independent of the surface of the collodion.

The size of the pores has been calculated from the vapor pressure of water in the collodion and from the rate of flow of water through the membrane. The results do not agree and are not consistent with the observed rates of penetration.

The relative rates of penetration of the gases bear no relation to the density of the gas.

When the results are corrected for the solubility of the substances in the collodion and expressed as the diffusion coefficient in collodion they show that the diffusion coefficient increases rapidly as the molecular weight decreases.

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PHYSIOLOGICAL ONTOGENY.

A. CHICKEN EMBRYOS.

XIV. THE HYDROGEN ION CONCENTRATION OF THE BLOOD OF CHICKEN EMBRYOS AS A FUNCTION OF TIME.

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These experiments continue to illustrate an interest in the processes of growth which are exhibited in earlier communications (1). We have turned now to the study of a more general function and have studied the changes which take place in the hydrogen ion concentration of the blood of chicken embryos. Chickens were selected in the expectation that the new data might form part of the information already accumulated and might serve the purpose of further inferences and wider generalization.

The ages of the embryos studied ranged between 8 and 20 days of incubation. It was impossible to secure sufficient volumes of blood before this age to permit measurements to be made, even when the vessels were successfully punctured by the needle of the syringe. We regret this omission from our data the more because we are aware of the fact that it is precisely at this early age, from 5 to 8 days, that information is desirable. It may be possible at another time to return to this period of the life cycle when methods other than those now available are at our disposal. During the period of hatching and in the first days of life, other difficulties beside that of drawing blood were encountered. Although no difficulty was experienced at the time of hatching in obtaining blood, it was found necessary to carry out this procedure either while the animals struggled or when they had been rendered quiet by the use of anesthetics such as amytal. In both cases, the blood must inevitably be unnatural as the result of dis-

turbance of the oxidation processes, due either to excess lactic acid formation during struggling, or in anesthesia to moderate asphyxia and to diminished rate of blood flow. We learned in the course of our experiments to avoid including measurements taken of the blood of embryos after they had been injured or when they were in a moribund state. In both cases there is a strong tendency for the blood to be more acid than is natural. We came in the end to recognize the presence of these states from examining the blood alone. It has occurred to us that the inclusion of such estimations might result in the publication of erroneous curves.

The incubation of the eggs of White Leghorn hens was arranged in the manner formerly described (2). The ages of the embryos were estimated with care, subject to the difficulties which are usually encountered even when eggs are brought promptly to the incubator after having been laid. The embryos were subsequently studied without removal from the incubator room. The glass electrodes (3), employed in measuring the hydrogen ion concentration, were also installed here. Embryos and their shed blood were, in short, constantly at $38^{\circ} \pm 0.5^{\circ}\text{C}$. The wires from the electrodes were led, appropriately sheathed, from the incubator room, through its wall to the adjoining room, where the electrometer was housed.

Blood was drawn either from a vein (arterial blood) or from an artery (venous blood) through a needle into the barrel of a syringe. Into the end of the syringe, a second one which contained the piston was fitted identical with the first. The syringe system from point of needle to piston was filled with paraffin oil. A second person manipulated the piston.

In opening the eggs it was necessary to avoid the occurrence of hemorrhage. Help toward this end was obtained by painting the shell membrane, after enough of the shell was chipped away, with paraffin oil. By this means two objects were attained; first, it was possible to locate blood vessels, and second, capillary hemorrhage was prevented. It is not certain how the effect in preventing hemorrhage took place nor whether the method was free from danger; capillaries may have closed because the membranes, coated with oil, became impermeable to the passage of gases. Usually the amount of the surface of the egg affected in this way was small. When, through

clearing the membrane, blood vessels became visible, it was often possible to insert a needle into them without further dissection—obviously a great advantage. Both oxygenated and reduced blood was obtained from embryos of all ages; in older embryos, both varieties were often drawn from the same animal.

Measurements of the reaction of the blood could be undertaken with volumes no greater than 0.25 cc. When withdrawn the blood

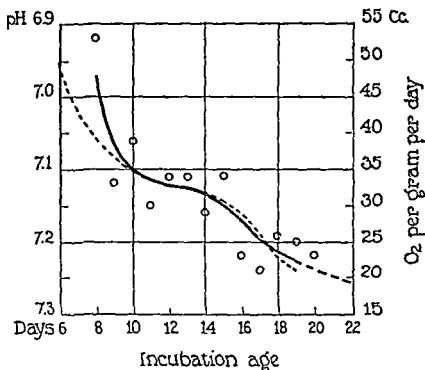


FIG. 1. A curve is shown (the solid line) indicating the course of the change in hydrogen ion concentration in the arterial (reduced) blood of chicken embryos. The circles represent the average pH of the blood at each day of incubation. In the middle of the curve, corresponding to the period between 10 and 14 days, there is a flattening of the curve. A curve (dotted line) published by Murray showing alterations with time of oxygen consumption, flattens at the same period; the ends of the two curves follow similar courses.

was plunged into Ringer solution already under oil in such proportions that the dilution of blood to solution was as 1 to 3 or 4. A small amount of sodium fluoride was likewise present in the test-tube to inhibit glycolysis. The effect of dilution to this extent in the value of the measurement, since it was not sufficiently known, has been studied. Preparations were made of undiluted and of diluted blood and estimated. The difference in value between the two was found

to be no more than a few hundredths of a pH. So diluted and prepared by vigorous stirring to prevent coagulation, the blood was transferred to the cup of the glass electrode. Measurements required so little time that the rate at which they could be made depended on the dissection of the embryos and the withdrawal of blood rather than on the manipulation of the apparatus.

We found a consistent change in the hydrogen ion concentration of the blood throughout that period of embryonic life which we examined. The general trend of the change was from acid to alkaline (Fig. 1 and Table I). At 8 days the pH averaged 6.92 and at 20 days 7.22. The lowest figure which was encountered was 6.75 in an 8 day embryo. We may point out that the lowest pH encountered so far in human beings is 6.95* and was found in a patient in a moribund state (4). It should be pointed out that the value in the venous blood of an adult animal, when the blood was withdrawn from an animal lying quite still without the use of anesthesia was 7.30. It is important to notice the low value in embryos of younger age. It suggests inferences that may be drawn from investigations published by Warburg and his collaborators (5) in connection with measurements of rapidly growing tissues in general, in the case either of tumors or of embryonic tissues in the period when their mass is undergoing great increase. We may recall that in this connection Warburg's measurements dealt with the rate of glycolysis measured in terms of the rate of lactic acid formation. There is to be found in this process not only increase in acid formation but actually an increase in the acid reaction. We are not in position to say that these two phenomena are necessarily mutually dependent. We shall return to this point.

We have for comparison with the results in fowl, studied the blood both arterial and venous, of the fetuses of cats. We were unable, unfortunately, to secure a continuous series so that data could be systematically collected according to time. The fetuses that were studied differed in age but the dating was too uncertain to allow us to draw a curve of the rate of change which takes place. The measurements which were made covered the range from pH 7.04 to 7.31. These it will be perceived lie within that found in chicken embryos. It

* In a personal communication Dr. D. D. Van Slyke tells us that this is the lowest pH of which he is aware.

appears that the reaction which is found in chicken embryos is in no way peculiar to this family, but is characteristic of embryonic and perhaps of rapid-growth in general. Okuneff (6) has in point of fact shown that plant tissue in the stage of regeneration after injury likewise exhibits a low pH.

TABLE I.

*The Changes in Hydrogen Ion Concentration of the Blood in Chicken Embryos.**

Incubation age <i>days</i>	Reduced blood		Oxygenated blood	
	Average pH	No. of observations	Average pH	No. of observations
8	6.92	13	7.03	8
9	7.12	4	7.18	5
10	7.06	6	7.23	3
11	7.15	4	7.19	1
12	7.11	4		
14	7.16	2	7.19	1
15	7.11	5		
16	7.22	4	7.22	3
17	7.24	3		
18	7.19	5	7.23	4
19	7.20	4	7.20	2
20	7.22	5	7.23	2
Adult	7.30		7.34	

* The standard deviations from the averages are not given. They are wider than would be encountered in the normal resting human subject. At the 8th day for instance, the range is 6.75 to 7.11. We have not given the figures in detail because in our opinion they are in a sense misleading. We believe the wide range is due to the impossibility not only of dating accurately the age of the embryos but also of excluding the possibility of injury. There seems now no reason to doubt that the pH of embryos, if they could be known to be of the same age, would be similar within a narrow range.

On our curve of change in the hydrogen ion concentration, we have inserted the points indicative of the change in oxygen consumption obtained by Murray (7) as expressive of change in the rate of metabolism. It is difficult to escape the impression that the two observe, while not a parallel, at all events a somewhat similar course. As a result of this comparison arises the suggestion either that both are

expressions of the same underlying process, or that they stand in some causal relation to each other. That oxygen consumption and lactic acid formation are both expressions of the metabolic function, is already known. The fact that the curve expressing the change in hydrogen ion concentration takes the form it does in respect to Murray's curve of oxygen utilization raises the question of the possibility of the existence of a relation between lactic acid production and the concentration of hydrogen ions. We are not in possession of measurements which place the formation of lactic acid in a determining position in respect to reaction but have been led to think of this possibility on account of the facts which have just been described.

SUMMARY AND CONCLUSION.

We have studied by means of glass electrodes the hydrogen ion concentration of the blood of chicken embryos from 8 to 20 days. When plotted as a curve, the average data show that a constant change takes place in the measurement, being acid at the beginning of this period and becoming alkaline toward its end. The acid reaction we think was characteristic not only of the embryos of fowl, but of the fetuses of cats and indeed as Warburg has shown of rapidly growing tissue in general. We have suggested the possibility, though we have no data to substantiate the suggestion, that the hydrogen ion concentration may under conditions like these, seeing that the curve of change resembles that of oxygen consumption, be expressive of changes in the rate of metabolism.

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MODIFICATION OF THE NORTHROP-KUNITZ MICROCATAPHORESIS CELL.

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A modification of the microcataphoresis cell described by Northrop and Kunitz has been used by the author for the study of many different types of suspensions and of different aspects of electrokinetic phenomena. (For a summary and literature review see Abramson: Colloid chemistry symposium monograph, No. VI, New York, 1928.) The essential differences in construction and use of the apparatus as used by the author are briefly described in the following and in Figs. 1 and 2.

1. The cell is not only made entirely of glass, as described by Northrop and Kunitz, but is so blown that the electrode vessels are fused to the cell itself.*

This is of advantage in that:

(a) The apparatus is smaller, lighter, more readily handled and is filled with no difficulty.

(b) It can be placed directly on the sliding stage of the microscope and can be held in position by two brass extensions which move with the stage.

(c) It is easily cleaned with cleaning mixture between measurements with no other manipulation than sliding the entire apparatus out of its support.

(d) If it be desired to resuspend particles which have fallen out, the apparatus is simply turned upside down.

2. Instead of Zn-ZnSO_4 reversible electrodes it has been found con-

* Two types of glass cells are available: (1) a cell of soft glass of uniform cross-section; (2) a cell of Pyrex or Jena glass drawn out sideways, but not of uniform cross-section. See article in Bibliography for further information.

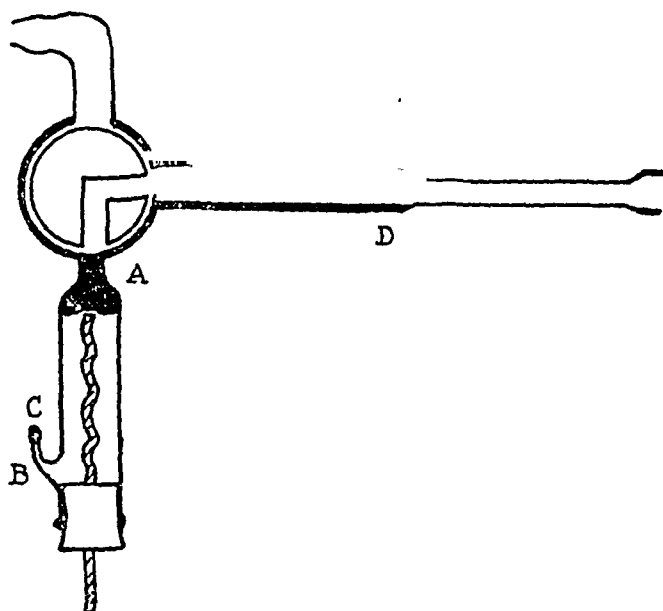


FIG. 1. Schema in longitudinal section of apparatus. At *A* is the agar plug *B* is the outlet, sealed off at *C*. The cell itself is fused to the electrode arm at *D*. By reference to previous publications, and Fig. 2, other details can be obtained

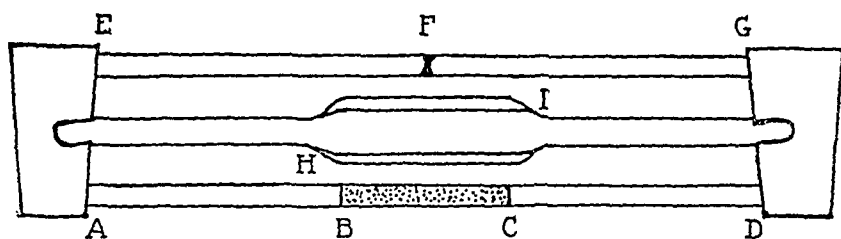


FIG. 2. Schematic view from above. The bars *ABCD* and *EFG* are solid glass supporting rods. Part of the bar *BC* (stippled) is curved so that dark-field illumination from the side is possible. Shaded *F* of the rear supporting rod is a break in *EFG* which is sealed with a suitable cement. By cementing glass plates above and below at the sides *H*, *I* of the cell itself, surfaces are obtained for both manual and mechanical manipulation.

venient to use the following systems, depending on the type of experiment to be made:

(a) Cu-CuSO_4 —electrolyte-free agar

- (b) Cu-CuSO₄-agar in isotonic saline solutions
- (c) Cu-CuSO₄-agar in saturated salt solutions like Na₂SO₄ and KCl.

The use of agar prevents streaming of the liquid from the electrode chamber into the system to be measured. In the case of suspensions in systems with high protein concentrations, as in serum, streaming occurs incidental to the heavy protein precipitate. This streaming, which makes measurement impossible, can be prevented by an agar plug. If the agar plug is electrolyte-free, it is best to allow freshly prepared electrodes to stand overnight before use. Otherwise the applied electrical force may move the agar, slightly, but sufficiently to cause turbulence. Saturated KCl agar has also been used. In the presence of the chloride ion Cu⁺⁺ is apparently reduced by the copper electrode to Cu⁺. Although the saturated KCl-agar electrode works excellently as a $\text{Cu} \rightleftharpoons \text{Cu}^+$ electrode, a precipitate on the copper may occasionally cause disturbance. Instead of the side arm stop-cocks with a ZnSO₄ reservoir as devised by Northrop and Kunitz, the capillary outlets in the diagram are situated as indicated. These facilitate filling the electrode chamber with CuSO₄ solution. They are sealed off with a suitable cement after stoppers are in place. A suitable diameter for the electrode vessels is 1 cm. The reproducibility of measurements is considerably enhanced by making consecutive experiments at the same point in the cell. Hairs are crossed and cemented to the top of the cell with Canada balsam. By using the point of crossing of the hairs as a reference mark, it is simple to return to the same point.

3. The calculations of the drop in potential may be accomplished as Northrop and Kunitz suggested. It has been found more convenient to use the following scheme. It follows from Ohm's law that E , the drop in potential per cm. in the cataphoresis cell is

$$E = \frac{IR}{q}, \quad (1)$$

where I is the current, R the specific resistance of the suspension and q the cross-section of the cell at the point of measurement. Since measurement of current prevents errors possible through accidental resistances, it is advisable to use the method of calculation as expressed in equation (1) when possible.

The movements of the water and particle within the cell follow the theory of von Smoluchowski in the case of small E.M.F's. If there is a very high electroendosmotic streaming of the medium, the curve of particle velocity may deviate from the well known parabolic form. This deviation is in the nature of a diminished return flow in the mid-regions of the cell, due perhaps to turbulence phenomena in other parts of the system rather than in the cell itself. Except for these anomalous and mostly avoidable cases, the curve is parabolic. In making measurements one should therefore use the lowest E.M.F. possible for the degree of accuracy required. When the curve of particle velocity at different levels is parabolic, the curve of velocity as plotted against level is the same near the fused ends of the cell itself (within 1.5 mm.) as in the middle. The stream lines of the liquid throughout the cell are therefore uniform. A most useful magnification is that obtained with a Zeiss 28 \times ocular and 40 \times water immersion objective. The working distance with this objective is 1.6 mm.

SUMMARY.

A modification of the Northrop-Kunitz microcataphoresis cell is described. Although the theory of von Smoluchowski relating to such systems is in general followed, certain deviations may be encountered which are easily avoided.

Addendum.—When the cell is used frequently, it may occur that the current passing through the system gradually falls. This indicates that there is a resistance at some point in the circuit. It can easily be shown that this resistance is at the junction of the agar plug and the solution in the cell itself. I have found that if the agar plug remains in contact with a saturated KCl solution for about 20 minutes, the constancy of the current flow is restored. It seems likely that a thin semi-insulating film of stop-cock grease on the agar surface may be responsible for such erratic behavior of the electrodes.

I am indebted to Professor L. Michaelis and to Professor H. Freundlich for valuable advice received in connection with this investigation.

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STUDIES ON PERMEABILITY OF MEMBRANES.

VII. CONDUCTIVITY OF ELECTROLYTES WITHIN THE MEMBRANE.

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The present communication deals with the results of measurements of the conductivity of the dried collodion membrane and with some accessory experiments which aid in interpreting the conductivity data.

The measurement of the conductivity of a membrane in equilibrium with an electrolyte solution presents certain difficulties which prevent determinations throughout all ranges of concentration and which limit the number of available electrolytes. Under the conditions of measurement the membrane forms only a small fraction of the whole path of the electric current and the conductivity of the membrane can be determined only when the resistance of all other parts of the system is relatively negligible or when the resistance of the other parts can be measured separately. The latter method is certainly the less dependable and is applicable only when the required correction is small. In collecting our data it turned out to be desirable to use two methods of making measurements, each method having its own field of application.

In the first method the ordinary potentiometer outfit used for recording potential differences was utilized. The applied E.M.F. in millivolts necessary to produce a standard deflection of the galvanometer was compared with the E.M.F. required to produce the same deflection when the membrane system was replaced by a known resistance. The membranes used were of the flat type previously described but in which the membrane was fixed to the end of a straight glass cylinder about 1 inch in diameter instead of over a bell jar. A measurement of the resistance was performed with a glass cylinder carrying no membrane, *i.e.*, with a free open end, and then with a membrane

in place, the electrolyte solution being the same in both cases. The difference between the two readings gave the resistance of the membrane. The membrane was kept constantly in the solution being investigated until readings successively constant over several days indicated that equilibrium had been attained. According to the nature of the solution one or several weeks were required for this purpose. The readings obtained under a given condition did not show any drift from the moment at which the electric current was closed, over a period of several minutes. So it is obvious that we are measuring real ohmic resistance and that no appreciable polarizing counterelectromotive force during the time of reading is established which might produce an additional apparent resistance.

This method proved to be suitable for recording the conductivity in solutions of neutral salts such as KCl and LiCl within a range of concentration from 1.0 N to about 0.01 N. In more dilute solutions the resistance of the solution itself was so high as to render correction unsatisfactory. It became evident that the resistance of the membrane was not a constant proportion of the resistance of the whole system. In high concentrations the membrane resistance was many times greater than that of the adjacent solutions. In very dilute solutions the two figures were of the same order of magnitude. On the other hand in the special case of HCl the membrane resistance was so low even in high dilutions as to make the required correction unsatisfactory. The results of these experiments carried out with eight different membranes have been brought together in Fig. I. The relative resistances of the membranes are plotted as ordinates; the abscissæ give the negative logarithm of the concentration. It will be seen that with HCl in all ranges of concentration the resistance is very low, so low that the relatively large corrections involved rob the figures of any absolute meaning. But it is evident in a striking way that the conductivity with HCl is enormously greater than with either KCl or LiCl. In the experiments with these latter salts it will be seen that the resistance with KCl is always much less than that with LiCl, that with both salts the resistance increases as the concentration decreases and that with neither is there proportionality between the resistance and concentration except, perhaps, in solutions more concentrated than $\frac{1}{3}$ normal. The experiments with very dilute solu-

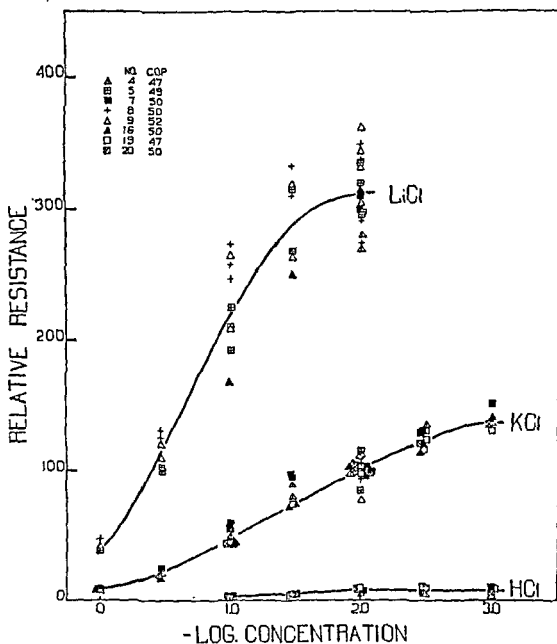


FIG. 1. Showing the relative resistances of the dried collodion membrane in contact with different electrolyte solutions as determined by the first method described in this paper. The experiments from which the figure was drawn were performed with eight different membranes; in most instances more than one determination was made. Since the resistances of these membranes under equal conditions differed widely (25,000–80,000 ohms in 0.01 N KCl) the figures have been brought within the scope of a single chart by setting the average resistance of each membrane in 0.01 N KCl equal to 100 and plotting the points proportionally.

tions have not been included in the chart because of the inaccuracies attending such determinations. Because of the same sources of error it is not desirable to consider the figures obtained as absolute values for the membrane resistances; nevertheless they will prove serviceable when regarded as approximations.

The second method of measuring the membrane resistance was essentially the use of a Kohlrausch bridge with an alternating current produced by a generator of 1,000 cycles. Compensation was effected with the aid of a telephone receiver. In producing a satisfactory tone minimum it was often necessary to balance the capacity involved in the membrane-electrolyte system by means of a variable condenser in the corresponding arm of the bridge. The membrane was one of the usual flat type prepared on a bell jar frame. After complete drying the membrane was cut out and sealed with collodion between two rubber washers. The whole was then clamped between the two halves of the conductivity cell (Fig. 2). Each half cell contained a circular platinum electrode so arranged that when the cell was put together its surface would lie within several millimeters of that of the membrane. The electrode was of such a size as just to allow space at its periphery for free circulation of the solutions under observation. The distance of the electrode from the membrane was as small as possible in order that the resistance of the liquid outside the membrane might form as small a part as possible of the resistance of the whole system. This condition is fulfilled only with membranes of high resistance and is of course totally inapplicable to the more usual type of collodion membrane. The electrolyte solution being investigated was renewed every day until the readings became constant. According to the nature of the solution 3 days to more than a week were necessary for this purpose. The results with any definite solution could be reproduced after some months except for the fact that all resistance data tended to become a little lower as time went on. Obviously the membrane permeability was a little increased in the course of several months. It is surprising that this deterioration was as slight as it was when the long contact with such solutions as 0.5 N HCl is considered.

This method also proved to be applicable only in limited ranges of concentration. Although no upper limit exists there is a very interesting lower limit. For example, with HCl when in the course of weeks

the concentration is lowered more and more a resistance is finally reached which no longer increases with decrease in concentration. Actually in 0.1 N solution a value of 7,150 ohms was obtained, in 0.01 N solution a value of 22,000 ohms and in 0.001 N solution 26,000 ohms. At this point the 0.001 N HCl solution was replaced by distilled water.

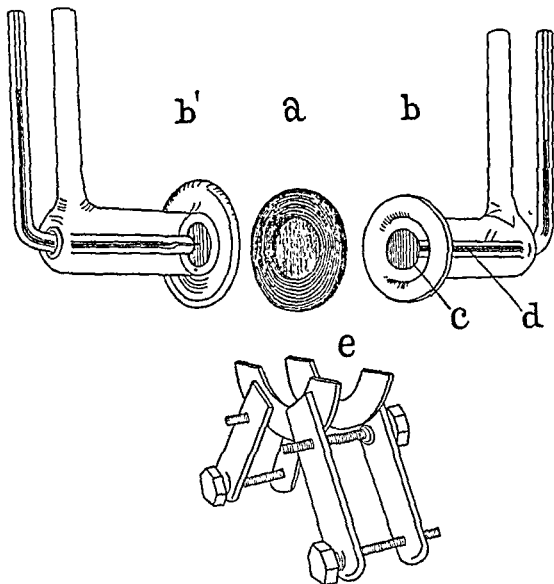


FIG. 2.

An immediate reading of 160,000 ohms was obtained but in the course of some hours this value had decreased to 30,000 ohms. It was then found that no matter how often fresh distilled water was introduced the final resistance of the system after some hours was the same—about 30,000 ohms. The only interpretation possible seemed to be that

some substance liberated by a slow process of disintegration within the membrane was fixing the level of resistance.

Experiments with other electrolytes, KCl, NaCl and LiCl, likewise disclosed a maximum level of resistance but these levels were quite different from each other and all different from that obtained with HCl. The conductivity of the membrane in 0.5 N NaCl was smaller than the limiting value for the HCl solutions regardless of the degree of dilution and that for LiCl was still lower (55,000 ohms). These observations have provided us with the criteria necessary to evaluate our results. They have led us to conclude that it is never justifiable to draw conclusions from conductivity data when the dilution of a particular electrolyte is such that the limiting resistance is approached. For in this case we are no longer dealing with the effect of dilution but with some inherent property of collodion itself.

The results of these experiments have been listed in Table I. With the above mentioned criteria in mind we can state that with HCl in the region of 1.0 N the conductivity is approximately proportional to the concentration. In higher dilutions proportionality no longer exists but because of the limiting resistance which is approached these figures have no satisfactory interpretation. With KCl the resistance is enormously greater than with HCl. Even in a solution as concentrated as 0.5 N a value of 20,000 ohms or more is reached. Although the limiting resistance value for a KCl solution is a little higher than for one of HCl, still the resistance even in the 0.5 N solution is so high that no accurate evaluation of conductivity is possible. But it can be stated that the membrane resistance in a 0.5 N KCl solution is at least 17 times as great as in an HCl solution of the same concentration and probably much more. In the same way we can conclude that the resistance of the same membrane in a 0.5 N LiCl solution is at least 40 times as great as in the corresponding HCl solution.

In reviewing the figures obtained by the two methods just outlined it is evident that the first method yielded little that was not more satisfactorily obtained by the second. In the second method the use of an alternating current assured the absence of even slight polarization effects and the closeness of the platinum electrodes to the membrane surface rendered the resistance of the solution surrounding the membrane negligible as compared with the membrane resistance.

It was thus possible to make measurements with HCl solutions which were not possible by the first method and we became conscious of the limiting resistance values,—values which must have modified the results obtained by the first method. However, because of the cost of the conductivity cell and the length of time (many months) required for a complete series of observations the figures obtained by the second method concern only one sample of membrane. By the first method we were able to work with a number of different samples of membrane. The results obtained by this method when interpreted in the light of the criteria established by the second are quite satisfactory for approximate estimations and show the variations with different specimens of membrane.

TABLE I.

Resistance as Determined by the Second Method (Alternating Current).

	Temperature 23°C.					
	Molar concentration of electrolyte					
	1	1/2	1/4	1/10	1/100	1/1,000
HCl	650	1,240		7,100	22,500	30,000
KCl		22,500	55,000	75,000		
NaCl		41,000				
LiCl		55,000				

A study of all the data obtained leads us to believe that in solutions more concentrated than $N/10$ HCl, $N/4$ KCl or $N/3$ LiCl the conductivity was approximately proportional to the concentration of the electrolyte. It is to be emphasized that this is not the case in the more dilute concentrations. Here, however, the diminishing mobility of the anion, as shown in previous communications, might have altered this proportionality appreciably. Furthermore it is evident that there is an enormous difference in the conductivity of different electrolytes, the conductivity decreasing in the order $\text{HCl} > \text{KCl} > \text{NaCl} > \text{LiCl}$, the differences being many times greater than if no membrane were present.

In attempting to interpret the data obtained we were faced with two questions. (1) Were the differences in conductivity due entirely

to differences in the specific mobilities of the various cations in the membrane? (2) Might not it be that the degree to which an electrolyte could enter the pores of a membrane depended on the nature of the electrolyte? This latter question was especially suggested by the results of our experiments on the diffusion of nonelectrolytes. It was here shown that the relatively slow diffusion of a large molecule, such as glucose, when compared with the many more rapid diffusion of a smaller molecule, such as acetone, was due to the fact that relatively few pores in the membrane were large enough to permit the entry of the larger glucose molecules.

In order to gather data which would enable us to decide between these possibilities it became desirable to determine the actual electrolyte content of membranes after thorough soaking in different electrolyte solutions. After numerous trials the following method was finally adopted. The chlorides of the various cations were selected because of the accuracy with which the electrolyte content could be determined by titration of chlorine. Relatively concentrated solutions (0.5 N) were used in order to obtain a sufficient quantity of electrolyte for satisfactory analysis. A membrane of the ordinary jar type was allowed to stand for several days in contact with a particular chloride solution under investigation in order that it might become saturated with the electrolyte solution. At the end of this time the membrane and bell jar were thoroughly washed with distilled water to remove all traces of surface solution. We do not believe that washing could remove any appreciable part of the electrolyte within the membrane pores because of the known fact that the diffusion of an electrolyte through these membranes against pure water is extremely slow. This belief was made certain by the analyses which showed the same electrolyte content whether the membrane was in contact with the washing water for 1 or several minutes. The membrane was now placed between two 0.1 N solutions of chloride and sodium nitrate and an electric current passed by means of platinum electrodes in such a direction that anions would migrate to the interior of the bell jar. During the first minutes of the experiment the anion carrying current would be Cl^- ; as the experiment continued more and less would be carried by Cl^- and more and more by NO_3^- . We therefore interrupted the experiment from time to time, intro-

fresh nitrate solutions and analyzed the solution from the anode compartment for its content of chlorine. This content became rapidly less with succeeding extractions. From the results we could plot a curve, the asymptote of which represented the original electrolyte content of the membrane for the solution being investigated. As an example

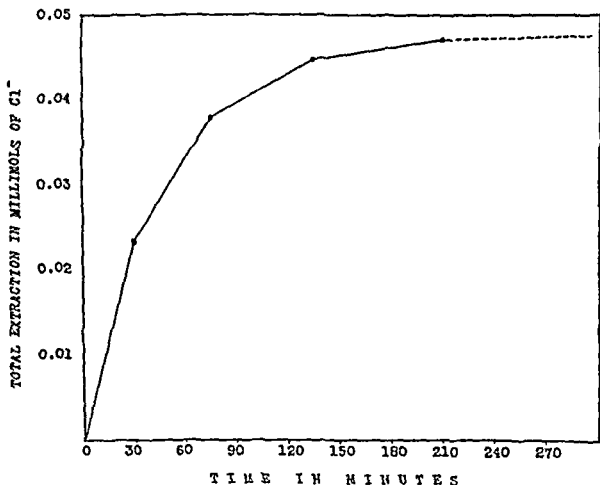


FIG. 3. This chart shows the result of one experiment in which the total electrolyte content of the membrane C-6 was determined after several days contact with a 0.5 *N* solution of KCl. Repeated extractions of Cl⁻ were made with a current of constant intensity (4×10^{-3} amperes), the sum of these values being plotted as the total Cl⁻ extraction in a given time. The asymptote of the curve represents the value of the electrolyte content.

one of these curves obtained with KCl is shown in Fig. 3. Several experiments were made with each solution in order to establish the constancy of the results. Two membranes were used,—one for comparing HCl and KCl, the other for contrasting KCl and LiCl. The results of individual experiments together with the average results

have been listed in Tables II and III. It will be seen that the electrolyte content of a membrane in equilibrium with 0.5 N HCl is approximately the same as with KCl. With LiCl the content is approximately half that with KCl.

DISCUSSION.

When we bring together the results of conductivity measurements and those concerned with the electrolyte content of the membrane it will be seen that with HCl and KCl there is a great difference in

TABLE II.

Electrolyte Content of Membrane C-6 in Contact with 0.5 N Solutions.

HCl	KCl
<i>millimols</i>	<i>millimols</i>
0.044	0.045
0.041	0.047

Results of experiments with one membrane in which the total electrolyte content was determined after several days contact with 0.5 N solutions of HCl and LiCl. The electrolyte content is essentially the same with the two electrolytes.

TABLE III.

Electrolyte Content of Membrane I-1 in Contact with 0.5 N Solutions.

KCl	LiCl
<i>millimols</i>	<i>millimols</i>
0.019	0.011
0.020	0.011
	0.010

Results of experiments with one membrane in which the total electrolyte content was determined after several days contact with 0.5 N solutions of KCl and LiCl. The electrolyte content of the membrane with KCl is almost the double of that with LiCl.

conductivity whereas the electrolyte content is essentially the same. In this case then we must conclude that the difference in conductivity is due almost entirely to differences in mobility. On the other hand with KCl and LiCl the differences in conductivity correspond fairly

well with the differences in the membrane electrolyte content,—the conductivity and electrolyte content with KCl being roughly the double of that with LiCl. Here it is apparent that no unusual change in mobility need be assumed to explain the result. Inasmuch as for the reasons already given conductivity measurements of the dried collodion membrane do not possess the highest order of accuracy and because the measurements of conductivity and those of the membrane electrolyte content were necessarily made with different samples of membrane, it is not desirable to treat the comparative results in a strictly quantitative way. Nevertheless they are quite sufficient for a few general conclusions concerning the behavior of these membranes with different electrolyte solutions.

An understanding of the facts just presented may be had by considering the same factors used for explaining the differences in the diffusion of non-electrolytes. The pores of the dried collodion membrane are not of uniform size and the membrane is therefore not uniformly permeable to molecular particles of different sizes. When dealing with strong electrolytes the participation of undissociated molecules can be neglected. An anion cannot migrate independently of its cation and therefore a given electrolyte can utilize only those pores large enough to allow the passage of the larger of its ions. Ionic volume is determined not by atomic weight but by the extent to which the particular ions are hydrated. We are aware that according to the Born theory the water shell surrounding an ion does not represent a fixed stoichiometric quantity, but nevertheless all of our concepts of the hydration of ions are based on supposed differences in the volume of the surrounding water shells. We may imagine the sphere of water which sticks so fast to the ion as to resist its separation with a definite force to be different for different kinds of ion. In so far we may speak of differences of volume of the water shell for different ions even though the magnitude of such a volume may not be strictly definable. The results of our experiments are quite in accord with the generally accepted ideas concerning the degree to which the various ions are hydrated. With HCl the H^+ ion is certainly not bigger than the hydrated Cl^- ion and the quantity of HCl entering a membrane is determined solely by the volume of the Cl^- ion. With KCl the volume of the K^+ ion is at least not greater than that of the Cl^- ion and

the latter again determines the quantity of electrolyte which can enter the membrane. In accord with this we found the membrane electrolyte content to be the same with HCl and KCl. On the other hand with LiCl the hydrated Li^+ ion is larger than the hydrated Cl^- ion and it is now the cation which will limit the quantity of electrolyte entering the membrane. In correspondence with this we found the membrane electrolyte content to be much less with LiCl than with either KCl or HCl.

Thus it would seem that two factors are operative in determining the specific conductivity of a membrane in equilibrium with a given electrolyte solution. In the first place the conductivity will depend upon the number of membrane pores large enough to allow the entry of the ions of the electrolyte. But even with different electrolytes which utilize the same membrane pores the ionic mobility and thus the conductivity, because of variations in specific frictional resistance, is much more highly dependent on molecular size than would be true if no membrane were present.

SUMMARY.

Two methods of measuring the electrical conductivity of the dried collodion membrane in contact with an electrolyte solution are described and the results of such measurements with different electrolytes in different ranges of concentration recorded. Some of the difficulties encountered in making these measurements are outlined. Of special interest was the fact that each membrane with each electrolyte showed a maximum level of resistance at a certain point in the dilution scale, a level which was not surpassed by further dilution. It is believed that this level was fixed by the collodion itself rather than by the contiguous electrolyte solution. Its existence limited the results available for reasonable interpretation. In relatively concentrated solutions the conductivity was shown to be approximately proportional to the concentration. With different electrolytes in the same concentration it was shown that the conductivities varied much more than in simple solutions without a membrane and that they fell in the order $\text{HCl} > \text{KCl} > \text{NaCl} > \text{LiCl}$.

A method was described whereby the electrolyte content of a membrane in contact with different chloride solutions could be determined.

It was shown that a membrane saturated with either 0.5 N HCl or 0.5 N KCl had practically the same total electrolyte content whereas the same membrane in contact with 0.5 N LiCl contained only half the quantity. These results were used in interpreting the conductivity data, the evidence presented strongly suggesting that two factors are operative in causing the widely divergent conductivities recorded with different electrolytes. The first factor depended on the quantity of electrolyte which can enter the membrane pores, a quantity dependent on the size of the pores and the volume of the larger of the two hydrated ions of the electrolyte. This factor was the chief one in determining the difference in conductivity between KCl and LiCl. The second factor was concerned with differences in the mobility of the various cations within the membrane brought about by friction between the moving ions and the pore walls. With KCl and HCl the quantity of electrolytes entering the membrane was in each case the same, being determined by the size of the larger Cl^- ion. The widely different conductivity values were explained as due to the changes in the mobility of the two cations within the membrane pores.

STUDIES ON PERMEABILITY OF MEMBRANES.

VIII. THE BEHAVIOR OF THE DRIED COLLODION MEMBRANE TOWARD BIVALENT CATIONS.

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Our previous studies of the behavior of the dried collodion membrane toward electrolytes have been made chiefly with the monovalent cations, H^+ , Li^+ , Na^+ and K^+ . We have reported the results of electric transfer experiments, conductivity measurements, potentiometric studies and measurements of the total electrolyte content of the membrane pores. During the course of these various studies a few observations were made concerning the behavior of some membranes with bivalent cations, chiefly Ca^{++} . The results of these observations were somewhat different from what we had expected and at the same time we did not feel able to explain them satisfactorily. Now, however, in the light of more fully developed ideas concerning the structure of the membranes the results begin to have a definite meaning and indeed provide additional evidence in favor of these ideas. We shall give first the experimental results and then discuss briefly their significance.

Experimental Results.

1. *The Potential Difference of Concentration Chains with the Chlorides of Bivalent Cations.*—In measuring the p.d. established across the dried collodion membrane with 0.1 N and 0.01 N solutions of calcium chloride, the results are at first exceedingly inconstant and only after a period of 3 to 4 days, during which time the solutions are frequently renewed, is it possible to obtain readings which are in any way constant. One must also be sure that the calcium chloride used is not contaminated with salts of any of the monovalent cations

as the relatively slight potential effects due to the former might be totally hidden by the effect of the latter. It is advisable to use a system of intermediary bridges such as has been previously described in order that the solutions in direct contact with the membrane may not be contaminated with KCl from the agar bridges. When these precautions are taken it is possible after some days to obtain successive readings which agree within 2 or 3 millivolts. This degree of accuracy is sufficient for our purposes although less than one can obtain with salts of the monovalent cations. The slight fluctuation of the results is due probably in part to the presence of small amounts of impurities, in part to the fact that the much higher resistance of the membrane in calcium chloride solution lowers the sensitivity of the galvanometer and in part to the lowered permeability of the membranes to the Ca^{++} itself, a feature which will be discussed later. In one membrane which had a previously determined *CoP* value of 49 millivolts (P.D. between 0.1 N and 0.01 N KCl solutions) a final result of about -15 millivolts was obtained. The negative sign is used because the more dilute of the solutions was negative instead of positive as in the case of the monovalent cations. This membrane exhibited essentially the same potential difference when placed between 0.1 N and 0.01 N solutions of barium chloride, namely about -14.5 millivolts. In another membrane whose *CoP* value was 46 millivolts the calcium concentration potential was about -13 millivolts. These figures are almost in agreement with that of -18 millivolts reported by Fujita and Michaelis for the same concentration chain without membrane. So the effect of the membrane upon the potential difference appears negligible in this case.

2. *Electric Transfer Experiments.*—The method of performing these experiments has been previously described. When the solutions on the two sides of the membrane are calcium nitrate and sodium chloride the current can be so arranged that the only ions within the membrane are Ca^{++} and Cl^- , migrating in opposite directions. In our experiments the chlorine entering the anode compartment was determined by titration with 0.01 N silver nitrate in the presence of potassium chromate as previously described and the calcium entering the cathode chamber estimated by the Kramer-Tisdall blood technique. In our first experiments performed with electrolytes in concentration of

0.02 N we were not able to obtain a satisfactory agreement between the total transfer as calculated from the current passed and that actually found by analysis. For this reason these figures cannot be used for a critical analysis but it was evident in all of the experiments that more of the current was transported by Cl^- than by Ca^{++} , this result being the reverse of that obtained with the monovalent cations. Here it will be recalled that the transfer number of the anion in all dilutions was less than 0.5 and that the greater the dilution the smaller it became.

The results of our experiments in 0.1 N concentration were more satisfactory because of a relatively good agreement between the total

TABLE I.

Experiment No.	Current intensity	Time	Total transfer by calculation	Chlorine transferred	Calcium transferred	Total transfer by analysis	Transfer number of anion
	milliamperes	min.	milliequivalents	millimols	milliequivalents	milliequivalents	$\frac{\text{Cl}^-}{\frac{1}{2}\text{Ca}^{++} + \text{Cl}^-}$
89	2	30	0.0373	0.0190	0.0180	0.0370	0.513
90	2	30	0.0373	0.0170	0.0156	0.0326	0.521
91	2	30	0.0373	0.0187	0.0146	0.0333	0.561
92	2	30	0.0373	0.0210	0.0146	0.0356	0.590
93	2	30	0.0373	0.0218	0.0152	0.0370	0.589

Results of electric transfer experiments with calcium; 0.1 N calcium nitrate in anode compartment; 0.1 N sodium chloride in cathode compartment.

transfers as calculated and as determined by analysis. These results have been listed in Table I. It will be seen that in every instance the transfer number, expressed in equivalents, of chlorine is close to 0.5 and in general tends to be a little greater than this. All of these experiments were performed with a single membrane (C-1) with a *CoP* of value of about 49 millivolts.

3. *Conductivity Measurements.*—The difficulties of obtaining reliable measurements of the conductivity of the dried collodion membrane and the limited range of concentration which provides figures suitable for reasonable interpretation have been discussed in a previous communication. It will be recalled that our comparative experiments were performed in 0.5 N concentration. The membrane resistance in

HCl was at least 15 times less than in KCl, the order of resistances being $\text{HCl} < \text{KCl} < \text{NaCl} < \text{LiCl}$. With CaCl_2 an exceptionally high resistance was encountered and the results obtained cannot be regarded as having any absolute meaning. Suffice it to say that the resistance of a membrane in CaCl_2 is much greater even than in LiCl.

4. *Total Electrolyte Content of Pores.*—The method of estimating the total electrolyte content of the membrane pores by making repeated extractions with the electric current has already been described. The results obtained by this method indicated that a membrane has essentially the same electrolyte content when immersed in a solution of KCl as in one of HCl, but that in a solution of the same concentration of LiCl this content is only half as large. The same sample of membrane used in these previously reported experiments was also tested in 0.5 N solution of CaCl_2 . Here a much lower total electrolyte content was obtained than with any of the chlorides of monovalent cations. It was found that the same membrane in contact with the CaCl_2 solution contained only 20 per cent as much chloride ion as when in contact with LiCl and only 10 per cent as much as when wetted with a KCl solution.

DISCUSSION.

By viewing together the several experimental items just outlined it is possible to obtain an understanding of the behavior of the dried collodion membrane toward the calcium ion and probably toward the bivalent ions in general. The exceedingly small electromotive forces arising from concentration chains indicate that in the case of CaCl_2 the equivalent mobilities of the anion and cation within the membrane pores are essentially the same. As this is the same condition which exists in a free aqueous solution when no membrane is present we may state that the membrane exerts no effect in altering the relative mobilities of the anion and the cation. The same conclusion may be drawn from the results of the electric transfer experiments in which the transfer number of the anion, expressed in terms of equivalents (not molarity), 0.5 or slightly greater corresponds closely with the slightly negative potential arising in concentration chains. A diffusion chain of $m/10$ and $m/100$ CaCl_2 should have, on

the assumption of the equivalent mobility of Ca to equalize the one of Cl, an E.M.F. of -18 millivolts (the more dilute solution being negative). The experimentally found value of -14 millivolts in presence of the collodium membrane shows that a statement saying that the equivalent mobilities of Ca and Cl are equal within the membrane, is legitimate to a rather fair degree of accuracy. So the effect of the membrane, as to lower the relative mobility of the anion, vanishes when the cation is bivalent. Here any membrane effect disappears. The question at once arises as to which of the two actions is responsible for the equality. (1) Has the membrane acted so as to decrease the mobility of calcium to the same extent as it decreases the mobility of chlorine in the case of the chlorides of the monovalent cations? If this explanation be the correct one we need suppose no unusual behavior of the anion. (2) In the presence of calcium, is the specific effect of the membrane in retarding the mobility of chlorine abolished? If this were the proper explanation equality of transfer numbers would result because the chloride ion would move faster in the presence of calcium than in the presence of monovalent cations. The extremely high electrical resistance of a membrane in equilibrium with a solution of calcium chloride, as measured in our conductivity experiments, indicates that the mobilities of both calcium and chlorine are very much decreased within the membrane and that the main action is to decrease the mobility of calcium rather than to increase that of chlorine.

The results of experiments in which the total electrolyte content of a membrane immersed in a solution of CaCl_2 was compared with the content when immersed in KCl and LiCl indicate that the main reason for the low mobility of calcium is not that the membrane exerts any specific action on individual cations and decreases their mobility within the pores but that the membrane is highly impermeable to the calcium ion itself. The explanation for this impermeability is undoubtedly the same as that which we have already advanced to explain the slow rate of diffusion of glucose as compared with glycerol and acetone. Only a small fraction of the total pore area is distributed among pores large enough to permit the passage of the calcium ion. This concept as applied to electrolytes has already been elaborated in connection with the discussion of the difference in membrane

conductivities in HCl, KCl, NaCl and LiCl. We pointed out that the volume of an ion must be regarded as including the surrounding water shell and that the number of membrane pores able to transport an electrolyte is determined by that one of its ions which has the greater volume: Inasmuch as the hydrated calcium ion is considerably larger than the hydrated ions of either hydrogen, potassium, chlorine, sodium or lithium it follows not only that the quantity of electrolyte (CaCl_2) able to penetrate the membrane will be determined by the volume of the hydrated calcium ion and be independent of the chlorine ion but also that the conductivity and total electrolyte content must be less than with any of the other electrolytes mentioned.

In a previous paper we have given our reasons for believing that a certain range of pore sizes exists throughout which there is a rapid change in the actual mobility of the anion. All experiences have tended to show that when the pores are relatively large in size the specific effect of the membrane in retarding the mobility of anions is abolished or rendered so small that it escapes observation. Now, in the case of calcium chloride it would be seen that the large size of the hydrated calcium ion allows the salt to pass through the membrane only by way of the largest pores and that these pores are too large to exert any appreciable effect on the mobility of the chloride ion. For this reason large potential differences in concentration chains are not observed and electric transfer experiments show that both the anion and the cation have transfer numbers close to 0.5, when expressed in equivalents.

SUMMARY.

A study of the behavior of the dried collodion membrane toward the bivalent calcium ion showed that:

1. There is almost no potential difference established across a membrane separating two calcium chloride solutions of 0.1 and 0.01 N concentrations.

2. The transfer numbers of chlorine and calcium, as measured in electrical transfer experiments, are both close to 0.5.

3. A sample of membrane in equilibrium with a solution of calcium chloride has an extremely high electrical resistance, greater than is

observed with solutions of the chlorides of any of the monovalent cations.

4. The total electrolyte content of a membrane in equilibrium with a solution of calcium chloride was only 20 per cent of that observed when the solution was lithium chloride and 10 per cent of that found when the solution was potassium chloride.

In explaining these various results it is supposed that (1), (2) and (3) are all the result of (4), that is, of the inability of the calcium ion to penetrate any but the largest of the membrane pores. As the total quantity of electrolyte able to penetrate the membrane is very small the electrical conductivity must also be very small. Moreover, the few larger pores that are large enough to transport the hydrated calcium ion are too large to exert any appreciable effect in decreasing the mobility of the anion. Thus the membrane has no effect in modifying the potentials established across concentration chains with CaCl_2 and the transfer numbers determined experimentally are what one would expect if no membrane were present.

ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life, and appeared simultaneously with Volumes IX, X, and XI.

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THE COMBINATION OF GELATIN WITH HYDROCHLORIC ACID.

II. NEW DETERMINATIONS OF THE ISOELECTRIC POINT AND COMBINING CAPACITY OF A PURIFIED GELATIN.

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(Accepted for publication, December 19, 1928.)

I.

Introduction.

About 6 years ago the writer¹ reported the results of hydrogen electrode titrations and conductivity titrations of gelatin with hydrochloric acid. These experiments led to the conclusion that 1 gm. of gelatin was capable of combining with 8.6 to 8.9×10^{-4} equivalents of HCl. The latter figure appeared to represent a maximum which was not exceeded even in the presence of a large excess of acid. In this earlier work the experimental data were corrected on the assumption that gelatin was isoelectric and combined with no measurable acid at pH 4.70. Hence the validity of the figure for the combining capacity is limited by this assumption, as well as by the fact that it was obtained only with gelatin obtained from one particular source (Peter Cooper's Glue Factory) and purified in one particular way (Loeb's method of washing).

The difference between purified gelatins of different origin was brought out by the work of Kraemer and Dexter² in their study of the effect of pH on the light-scattering capacity of gelatin sols and gels. They assumed the pH where this effect was at a maximum to be

¹ Hitchcock, D. I., (a) *J. Gen. Physiol.*, 1921-22, iv, 733; (b) *J. Gen. Physiol.*, 1923-24, vi, 95; (c) *J. Gen. Physiol.*, 1922-23, v, 383; (d) *J. Gen. Physiol.*, 1923-24, vi, 201.

² Kraemer, E. O., and Dexter, S. T., *J. Phys. Chem.*, 1927, xxxi, 764.

that of the isoelectric point, and found this pH to be close to 5 for hide gelatins, 5.5 for an ossein gelatin, and 8 for a pigskin gelatin. They concluded that previous estimates of the isoelectric point of hide gelatins were too low, and showed that many of the data of previous workers were not inconsistent with an isoelectric point at pH 5.

The combining capacity of "Difco" gelatin, purified by Loeb's method, was found by Chapman, Greenberg, and Schmidt³ to be 10.4×10^{-4} equivalents of acid dye per gram of gelatin. They suggested that the writer's lower figures were due to the fact that the second basic groups of lysine, arginine and histidine were only 80 to 90 per cent dissociated (combined with H^+) in HCl even at pH 1. If this is true for these groups in gelatin, the curve of combined H^+ in HCl should not be horizontal between pH 2 and 1, but still ascending.

The present paper records an attempt to locate more exactly the isoelectric point of a gelatin from the same source as that used in the earlier experiments, to determine whether or not its combination curve with hydrogen ion is horizontal between pH 2 and 1, and to determine as exactly as possible the extent of its combining capacity for each of the ions of hydrochloric acid. The latter determinations have been made by electromotive force measurements of a cell without liquid junction, of the type used in exact thermodynamic studies in inorganic chemistry, but not previously used, so far as the writer is aware, in studies of protein solutions.

II.

Isoelectric Point.

The gelatin used in this work was part of a single 5 pound package of Cooper's gelatin, purchased from Peter Cooper's Glue Factory, Gowanda, N. Y. It was purified in 50 gm. lots by Loeb's method of washing, as improved by Northrop and Kunitz.⁴ It was dried by alcohol and ether in the air. Solutions containing about 13 per cent gelatin by weight were prepared by dissolving 50 gm. of this air-dry preparation in 250 cc. of distilled water. The exact concentration of each stock solution was obtained by weighing the solution delivered at 40° by a 10 cc.

³ Chapman, L. M., Greenberg, D. M., and Schmidt, C. L. A., *J. Biol. Chem.*, 1927, lxxii, 707.

⁴ Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1927-28, xi, 477.

pipette, and evaporating to dryness another sample delivered by the same pipette under the same conditions. Dry weights were considered constant when they decreased less than 0.2 per cent per day, which was the case after 5 to 15 days at 110°C. This gelatin was analyzed for total nitrogen by the Kjeldahl method, and found to contain 18.0 per cent N on a dry weight basis. Its ash content was found to be 0.04 per cent, which may be compared with the value 0.1 per cent obtained⁵ for gelatin purified by Loeb's original method. Its freedom from electrolytes may also be inferred from the conductivity data given in Table I.

The isoelectric point of the gelatin used in this work was determined by two methods, the pH of minimum osmotic pressure and the pH of

TABLE I.
Specific Conductivity at 30° of Purified Gelatin in Distilled Water.

Concentration of gelatin, gm. per 1000 gm. H ₂ O	Specific conductivity, 10 ³ × reciprocal ohms		
	Solution	Water	Difference
52.1	2.98*	0.12*	2.86
89.3	3.94*	0.12*	3.82
156.0	5.59†	0.58†	5.01

* Twice distilled water.

† Once distilled water.

maximum turbidity. Osmotic pressure measurements were made at 30°C., essentially as described by Loeb,⁶ using 1 per cent and 2 per cent gelatin solutions whose pH was varied by very small additions of HCl or NaOH. The differences in level of the inside and outside solutions were measured to the nearest millimeter after 1 or 2 days. Each reading was corrected by subtracting the capillary rise of water in the tubes used, about 6 mm. The concentration of gelatin inside each membrane was determined by dry weight determinations, and the results were brought to a common basis by dividing the corrected height in millimeters of solution by the concentration of gelatin in grams per kilo of water. The pH values were obtained with bubbling hydrogen electrodes at 30°C., and are based on Scatchard's value⁷

⁵ Loeb, J., *J. Am. Chem. Soc.*, 1922, xlv, 213.

⁶ Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1st edition, 1922; 2nd edition, 1924.

⁷ Scatchard, G., *J. Am. Chem. Soc.*, 1925, xlvii, 696.

4.7, but indicate that this sample of gelatin was isoelectric at pH 5.02 ± 0.05 .

Confirmation of this location of the isoelectric point of the gelatin used was obtained from observations of the effect of pH on the opacity of gels. The pH was varied by small additions of HCl or NaOH, and measured, as described above, at 30° . No attempt was made to get quantitative photometric measurements of the scattered light, but the gel of maximum turbidity was located by inspection of 10 cc. samples in test-tubes after standing overnight in a refrigerator at about 8°C . The results are given in Table II. Evidently for this gelatin the pH of maximum opacity is 5.05 ± 0.05 . It appears to be independent of the concentration of the gelatin, and to differ very little from the pH

TABLE II.
Effect of pH on the Opacity of Gelatin Gels.

Concentration of gelatin, gm. per 1000 gm. H_2O	Composition of solvent	pH of maximum opacity	pH of next samples in series
2	0.00005 N HCl	5.05	4.95, 5.11*
10	0.0001 N HCl	5.03	4.93, 5.06*
20	H_2O	5.07*	4.99, 5.17

* pH of gelatin + water.

of the gelatin in water, 5.08 ± 0.03 , which also seems to vary in no regular way with the concentration. Hence the isoelectric point of this gelatin may be taken as pH 5.05 ± 0.05 , and any correction for acid or base contained in the purified gelatin is so small as to be safely neglected in the calculations of combining capacity reported below.

III.

Combining Capacity for Hydrogen Ion.

The curves previously obtained by the writer^{1a,c} for the amount of hydrogen ion bound by gelatin as a function of pH are open to criticism because of the scattering of the points between pH 1 and 2. This scattering was due to the fact that, for 1 per cent solutions, the quantity plotted was a small difference between two larger concentrations of acid, each of which might be affected by the error inherent in a pH

determination. Hence the percentage error in the calculated amount of bound acid was often unreasonably large. It seems likely that such errors may account for some of the peculiar combination curves of protein with acid obtained by others.⁸ In the present work it has proved possible to reduce this error by using higher concentrations of gelatin.

The solutions were made up by weight from hydrochloric acid solutions standardized by Na_2CO_3 and AgCl , and gelatin solutions standardized by dry weight determinations at 110°C . The pH determinations were made in wide test-tubes, using platinized wire electrodes and bubbling hydrogen. Contact was made with a saturated KCl calomel electrode through bridges of saturated KCl in 3 per cent agar-agar jelly. The bridge tubes were drawn out and bent up at the end to minimize diffusion of KCl into the solutions. The bridges were dipped into the solutions only while readings were being taken. The E. M. F. of the cells was measured to 0.1 millivolt with a Leeds and Northrup Type K potentiometer. In some cases 2 or 4 hydrogen electrodes were used with samples of the same solution in different tubes; in other cases a single electrode was used with a given solution, but the cell was refilled and the measurement repeated. Readings were considered constant when they checked within 0.5 millivolt (often less) and showed no drift over a period of 20 or 30 minutes. The E. M. F. readings were corrected to 1 atmosphere of hydrogen by the tables of Clark.⁹ The cells were kept at $30^\circ \pm 0.02$ by an electrically controlled water bath.

The pH values were based on the standard mentioned above. Repeated measurements with a single calomel electrode (not made with especially purified materials) against hydrogen electrodes in 0.1 M HCl gave the E_0 value for this cell at 30° as 0.2405, and the pH values were obtained from the Nernst formula in the form

$$\text{pH} = \frac{E - 0.2405}{0.06015}.$$

⁸ Lloyd, D. J., and Mayes, C., *Proc. Roy. Soc. London, Series B*, 1922, xciii, 69. Hoffman, W. A., and Gortner, R. A., in Holmes, H. N., Colloid symposium monograph, New York, 1925, ii, 209.

⁹ Clark, W. M., The determination of hydrogen ions, Baltimore. 2nd edition, 1922.

TABLE III.

Determination of H⁺ Combined with Gelatin from Hydrogen Electrode Measurements with KCl-agar Junction.

<i>m</i>	<i>g</i>	<i>E</i>	pH	<i>a_H</i>	<i>γ_H</i>	<i>m_H</i>	<i>b_H</i>	$\frac{b_H}{g} \times 10^4$
0	2	0.5470	5.11	7.8×10^{-8}	1.000	7.8×10^{-8}	0	0
0	10	0.5445	5.06	8.7×10^{-8}	1.000	8.7×10^{-8}	0	0
0	20	0.5455	5.07	8.5×10^{-8}	1.000	8.5×10^{-8}	0	0
0	40	0.5445	5.06	8.7×10^{-8}	1.000	8.7×10^{-8}	0	0
0.01029	31.3	0.4850	4.07	8.6×10^{-8}	0.921	9.3×10^{-8}	0.01020	3.26 ± 0.00
0.0550	90.0	0.4525	3.52	3.0×10^{-4}	0.860	3.5×10^{-4}	0.0546	6.07 ± 0.00
0.02058	31.4	0.4425	3.36	4.4×10^{-4}	0.895	4.9×10^{-4}	0.02009	6.40 ± 0.00
0.0543	70.4	0.4270	3.10	7.9×10^{-4}	0.861	9.2×10^{-4}	0.0534	7.58 ± 0.00
0.0844	90.2	0.3910	2.50	0.0032	0.846	0.0037	0.0807	8.95 ± 0.00
0.0531	51.8	0.3790	2.30	0.0050	0.861	0.0058	0.0473	9.13 ± 0.02
0.0830	70.1	0.3510	1.84	0.0146	0.847	0.0172	0.0658	9.39 ± 0.04
0.0521	34.2	0.3465	1.76	0.0174	0.852	0.0204	0.0317	9.27 ± 0.15
0.1131	89.5	0.3375	1.61	0.0245	0.839	0.0292	0.0839	9.37 ± 0.07
0.0816	51.6	0.3335	1.55	0.0282	0.847	0.0333	0.0483	9.36 ± 0.16
0.1052	71.7	0.3300	1.49	0.0324	0.841	0.0385	0.0667	9.30 ± 0.13
0.1110	70.3	0.3255	1.41	0.0389	0.840	0.0463	0.0647	9.20 ± 0.16
0.0795	34.7	0.3245	1.40	0.0398	0.848	0.0470	0.0325	9.37 ± 0.32
0.1046	56.1	0.3225	1.36	0.0437	0.841	0.0519	0.0527	9.39 ± 0.22
0.1087	51.6	0.3190	1.30	0.0501	0.840	0.0597	0.0490	9.51 ± 0.26
0.1035	41.2	0.3160	1.25	0.0562	0.841	0.0668	0.0367	8.91 ± 0.36
0.1062	34.7	0.3135	1.21	0.0617	0.840	0.0735	0.0327	9.43 ± 0.46
0.1019	27.3	0.3120	1.19	0.0646	0.841	0.0768	0.0251	9.20 ± 0.65
0.2310	121.2	0.3005	1.00	0.1000	0.836	0.1196	0.1114	9.19 ± 0.23
0.2192	73.0	0.2935	0.88	0.1318	0.836	0.1576	0.0616	8.44 ± 0.49

m = molality of HCl.

g = gm. gelatin per kilo H₂O.

E = observed E.M.F. in volts, corrected to 1 atm. H₂.

$$\text{pH} = \frac{E - 0.2405}{0.06015}.$$

a_H = activity of H⁺ = antilog (−pH).

γ_H = activity coefficient of H⁺, from Scatchard's data.

$$m_H = \frac{a_H}{\gamma_H} = \text{molality of free H}^+.$$

b_H = molality of combined H⁺ = *m* − *m_H*.

$$\frac{b_H}{g} \times 10^4 = \text{mols of H}^+ \text{ combined with 10,000 gm. gelatin.}$$

The numbers following the ± sign represent the effect of an error of ± 0.5 millivolt in *E*.

The amounts of combined hydrogen ion were calculated by the method used by Cohn,¹⁰ which differs slightly from that used by Loeb⁶ and by the writer¹ in previous work. If b_H = molality of bound H^+ , m = total molality of HCl , and a_H = activity of H^+ as given by pH measurements, then

$$b_H = m - \frac{a_H}{\gamma_H}.$$

Here γ_H was taken as the activity coefficient of H^+ , according to Scatchard⁷, in a pure HCl solution of molality m . This is equivalent to assuming the ionic strength principle of Lewis and Randall¹¹ and assigning to the gelatin- H^+ complex an effective valence of one. In the method of calculation previously used the terms m and b_H represented volume normal concentrations and γ_H was taken as the activity coefficient of H^+ in that concentration of pure acid having a measured hydrogen ion activity equal to that found for the gelatin- HCl solution. This was equivalent to assuming that the gelatin chloride present had no effect on the activity of H^+ in the excess HCl . The present assumption seems more reasonable than that formerly used because of the results with acid-salt mixtures cited by Lewis and Randall.¹¹

The values of b_H were divided by the concentrations of gelatin to get the number of equivalents of H^+ combined with unit weight of gelatin. This quantity is given in the last column of Table III, and is plotted against pH in Fig. 2. The curve in Fig. 2 was drawn to represent the best smooth relation between the experimental points. It will be noted that the points do not show any evidence of a further upward trend with decrease of pH from 2 to 1, as would be expected if the explanation proposed by Schmidt³ and his co-workers were correct. Except for two points which lie below the curve, the points lie fully as close to a horizontal line between pH 2 and 1 as could be expected from the effect of a possible error of 0.5 millivolt in the E.M.F. of the cell. Although in many cases the agreement of duplicates was

¹⁰ Cohn, E. J., *Physiol. Rev.*, 1925, v, 349.

¹¹ Lewis, G. N., and Randall, M., *J. Am. Chem. Soc.*, 1921, xliii, 1112; *Thermodynamics and the free energy of chemical substances*, New York and London, 1923.

better than this, the measurements with HCl alone differed so much that the precision is probably not better than ± 0.5 millivolt or ± 0.01 pH for these agar bridge measurements.

The experiments thus confirm the writer's earlier conclusion that the amount of H^+ bound by 1 gm. of gelatin is constant between pH 2 and 1. For this gelatin this quantity is 9.35×10^{-4} equivalents of H^+ per gram of gelatin. This figure was confirmed by conductivity titrations of gelatin with HCl, carried out by the method previously

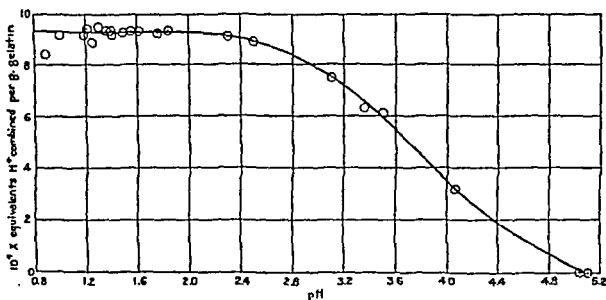


FIG. 2. The combination of gelatin with hydrogen ion from HCl. Ordinates are equivalents of H^+ combined with 1 gm. of gelatin, multiplied by 10^4 , as calculated from hydrogen electrode measurements with an agar-KCl junction. Abscissæ are pH values of the gelatin-HCl solutions, obtained from the same measurements. The points indicate a maximum of 9.35×10^{-4} equivalents of H^+ combined with 1 gm. of gelatin between pH 2 and 1.

described.^{1,2} The end-points of three titrations, using different concentrations of gelatin, gave the following results: 9.39, 9.37, 9.47×10^{-4} ; average, 9.41×10^{-4} equivalents per gram.

The difference between these values and those obtained earlier (8.9 by hydrogen electrode, 8.6 by conductivity) is more than accounted for by the different values assigned to the isoelectric point. Such differences might well be due to the very doubtful uniformity of the original commercial gelatin. It should be possible to avoid such differences and uncertainties by the use of gelatin prepared from definite materials in a definite way. A committee of the Leather and

Gelatin Division of the American Chemical Society has proposed tentative specifications for a standard gelatin for scientific purposes, and the writer hopes to be able to report later on the acid-combining capacity of gelatin prepared in accordance with these specifications.

IV.

Combination with Hydrogen and Chloride Ions.

A recalculation of old data^{1,c} and a few new experiments with silver-silver chloride electrodes in gelatin-HCl solutions seemed to indicate that gelatin combined to some extent with chloride ion, with a maximum combining capacity of 1.5 to 2.5×10^{-4} equivalents of Cl^- per gram of gelatin. It seemed possible to determine more exactly this combination with chloride ion, as well as that with hydrogen ion, by measurements of the cell without liquid junction: Ag , AgCl , $\text{HCl} + \text{gelatin}$, H_2 .

Four silver-silver chloride electrodes were prepared by electroplating, as in the previous work.^{1,c} They were replated whenever any two differed by 0.2 millivolt in the same solution, 0.1 M HCl . The hydrogen electrodes were short coils of platinized platinum wire, and were completely immersed in the solutions, with hydrogen bubbling up around them. Commercial tank hydrogen was used. Its purity was tested by hydrogen electrode measurements in 0.1 M HCl with hydrogen passed through alkaline permanganate, alkaline pyrogallate, and distilled water. Identical readings were obtained with only the washing in water at 30° ; accordingly the rest of the purifying train was omitted in the experiments. The vessels used were simple U-tubes about 2 cm. wide in the vertical arms and 1 cm. at the bend. A few measurements were made with the AgCl and H_2 electrodes in a single test-tube, but the E.M.F. so obtained decreased with time. The U-tubes, by protecting the AgCl from H_2 , gave readings which were constant to 0.2 millivolt for several hours. In each experiment 4 cells were set up with the same solution, and readings taken to the nearest 0.05 millivolt for 3 to 5 hours. The E.M.F. values were plotted against time and an average value selected from those points which could best be represented by a horizontal line over a period of at least 1 hour. The values so obtained are probably reliable to 0.1

millivolt or better as a measure of the true E.M.F. in any one experiment. Many experiments had to be discarded because of lack of constancy and reproducibility in the E.M.F. It was found in some cases that the erratic behavior was due to toluene which had been used to protect the stock solution of gelatin. In the course of repeated meltings and settings of the gelatin, the toluene seemed to become permanently emulsified. Other solutions which had been in contact with thymol gave low and rapidly drifting E.M.F. values, an effect which was reproduced with HCl containing thymol. The data reported below were obtained with gelatin to which no preservative was added. The readings were obtained with AgCl electrodes which had not been freshly plated for 2 or 3 months, but had been kept in 0.1 M HCl. After each day's use in a gelatin solution they were washed with hot water, distilled water, and 0.1 M HCl. At the end of this series of experiments the E.M.F. obtained with these electrodes in the cell Ag, AgCl, HCl (0.1 M), H_2 was just as constant and reproducible as at the start of the series, but was 0.6 millivolt lower. This lowering was either a slow time effect or else an effect of the contact with gelatin. The calculations were based on this final HCl reading as a standard rather than on the initial reading, although the latter was again reproduced on replating the electrodes.

The data and calculations are given in Table IV. Each figure is the mean of two experiments, each done with four sets of electrodes. The E.M.F. of the cell Ag, AgCl, HCl, H_2 at 30°C. is given by the thermodynamic equation

$$E = E_0 - 0.06015 \log a_H a_{Cl} \quad (1)$$

in which a_H and a_{Cl} represent the activities of the ions and E_0 is the E.M.F. of a hypothetical cell in which the product $a_H a_{Cl}$ is equal to one. The determination of E_0 by extrapolation has been discussed elsewhere.¹² For the present purpose it is not necessary to know the absolute value of this quantity. Equation (1) may be written

$$E = E_0 - 0.1203 \log \gamma - 0.06015 \log m_H m_{Cl} \quad (2)$$

where γ is the geometrical mean activity coefficient of the two ions and m_H and m_{Cl} are their respective molalities. This form of equation

¹² Hitchcock, D. I., *J. Am. Chem. Soc.*, 1928, 1, 2076.

has been applied to the calculation of the activity coefficient of HCl in the presence of other chlorides. The results of Harned,¹³ as quoted by Lewis and Randall,¹¹ show that in mixtures of HCl with LiCl, NaCl,

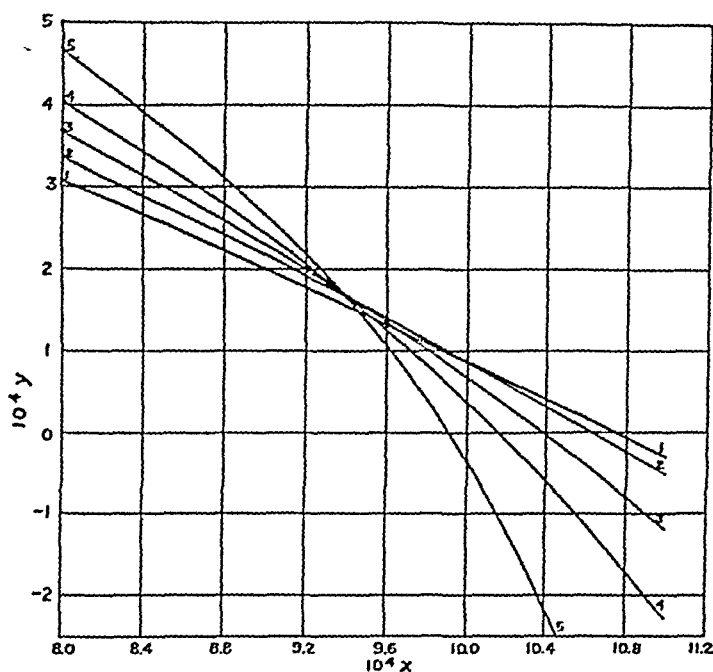


FIG. 3. Graphical determination of the combining capacity of gelatin with H^+ and Cl^- from E.M.F. measurements of the cell $Ag, AgCl, HCl + \text{gelatin}, H_2$, without liquid junction. Each experiment leads to an equation containing two unknowns, x and y , the equivalents of H^+ and Cl^- combined with 1 gm. of gelatin. In the figure the abscissæ are values of $10^4 x$ and the ordinates are $10^4 y$. Each curve represents the relation between these variables for one experiment, and is marked with the number of the experiment. The true values of these quantities, if constant for all the experiments, should be given by a single intersection of all the curves. The figure shows that four of the five curves intersect at the point where $x = 9.4 \times 10^{-4}$ and $y = 1.7 \times 10^{-4}$.

or KCl, at a constant total molality of 0.1 or 0.2, the activity coefficient is the same as that in pure HCl of the same total molality. The writer has confirmed this by experiments with 0.1 M HCl partly neutralized by NaOH, KOH, or NH_4OH . Accordingly in the calcula-

¹³ Harned, H. S., *J. Am. Chem. Soc.*, 1920, xlii, 1808.

tion of the gelatin experiments it will be assumed that the same principle applies; in other words, the partial neutralization of dilute HCl by gelatin is assumed not to affect the mean activity coefficient of its ions. On this basis the expression $E_0 - 0.1203 \log \gamma$ is assumed constant and equal to the value obtained without gelatin. An experiment done at the conclusion of the gelatin series with 0.1008 M HCl gave $E =$

TABLE IV.

Combining Capacities of Gelatin for H^+ and Cl^- from E.M.F. of Cells without Liquid Junction.

Experiment No.	m	g	E (observed)	$\frac{E - 0.2305}{0.06015}$	$m_H m_{Cl}$	E (calculated)
1	0.1005	13.5	0.3546	2.063	0.00865	0.3547
2	0.1014	27.3	0.3589	2.135	0.00733	0.3589
3	0.1025	44.5	0.3652	2.239	0.00577	0.3652
4	0.1036	59.8	0.3721	2.354	0.00443	0.3721
5	0.1048	77.4	0.3827	2.530	0.00295	0.3828

m = molality of HCl.

g = concentration of gelatin in gm. per kilo H_2O .

E (observed) = E.M.F. in volts of cell Ag, AgCl, HCl + gelatin, H_2 (1 atm.).

0.2305 = $E_0 - 0.1203 \log \gamma$, from E (observed) = 0.3504 for 0.1008 M HCl without gelatin.

$m_H m_{Cl} = \text{antilog} \frac{0.2305 - E}{0.06015} = \text{product of molalities of ions.}$

E (calculated) = $0.2305 - 0.06015 \log (m - gx)(m - gy)$ where m and g have values in columns 2 and 3, $x = 9.4 \times 10^{-4}$, and $y = 1.7 \times 10^{-4}$. The latter values were obtained graphically in Fig. 3 from the values of $m_H m_{Cl}$ in column 6 and the relation $m_H m_{Cl} = (m - gx)(m - gy)$.

0.3504. From this it follows that $E_0 - 0.1203 \log \gamma$, which is equal to $E + 0.1203 \log m$, is 0.2305. Now equation (2) may be rewritten

$$\frac{E - 0.2305}{0.06015} = -\log (m - gx)(m - gy) \quad (3)$$

where x and y are numbers of equivalents of H^+ and Cl^- combined with 1 gm. of gelatin, and g is the concentration of the gelatin in grams per kilo H_2O . For each experiment E , m , and g were measured; hence the equation contains the two unknowns x and y . The concentrations in the experiments were such that x and y might be expected to be constant,

the gelatin being combined to the maximum possible extent with both H^+ and Cl^- . This constancy may be tested, and the equation solved for both x and y , by plotting y as a function of x for each experiment. This was done by using the observed values of E , m , and g , assuming values for x , and calculating y . If the values of x and y are really constant, all the curves should intersect in a single point whose coordinates are the true values of x and y . The curves in Fig. 3 were obtained from the data of Table IV in this way, and the validity of the assumed constancy of x and y is justified by the single intersection of four curves. The form of equation (3) is such that algebraically x and y are interchangeable. The experiments in the preceding part of this paper indicate that the higher value belongs to x , the equivalents of H^+ combined with 1 gm. of gelatin. The experiments therefore lead to the conclusion that 1 gm. of gelatin in 0.1 M HCl combines with a maximum of 9.4×10^{-4} equivalents of H^+ and 1.7×10^{-4} equivalents of Cl^- . The consistency of these figures with the experimental data is shown by the last column in Table IV, which gives the E.M.F. calculated by equation (3), using the observed values of m and g and the above values of x and y . The agreement of these with the observed values shows that the failure of Curve 1 in Fig. 3 to intersect the others at a single point is not due to an unreasonable experimental error in the E.M.F., but simply due to the magnification of error inherent in an experiment with a low protein concentration. These figures are probably more reliable than those obtained from the agar bridge data because of the absence of liquid junction errors in these cells. It is hoped that this method may be used later in a study of the standard gelatin mentioned above, as well as with other proteins.

V.

Summary.

1. Cooper's gelatin purified according to Northrop and Kunitz exhibited a minimum of osmotic pressure and a maximum of opacity at $pH\ 5.05 \pm 0.05$. The pH of solutions of this gelatin in water was also close to this value. It is inferred that such gelatin is isoelectric at this pH and not at $pH\ 4.70$.

2. Hydrogen electrode measurements with KCl-agar junctions were

made with concentrated solutions of this gelatin in HCl up to 0.1 M. The combination curve calculated from these data is quite exactly horizontal between pH 2 and 1, indicating that 1 gm. of this gelatin can combine with a maximum of 9.35×10^{-4} equivalents of H^+ .

3. Conductivity titrations of this gelatin with HCl gave an endpoint at $9.41 (\pm 0.05) \times 10^{-4}$ equivalents of HCl per gram gelatin.

4. E.M.F. measurements of the cell without liquid junction, Ag, AgCl, HCl + gelatin, H_2 , lead to the conclusion that this gelatin in 0.1 M HCl combines with a maximum of 9.4×10^{-4} equivalents of H^+ and 1.7×10^{-4} equivalents of Cl^- per gram gelatin.

The writer wishes to acknowledge the faithful assistance of Miss Esther R. Mason, who did most of the experimental work reported in this paper.

CHEMICAL ANTAGONISM OF IONS.

III. EFFECT OF SALT MIXTURES ON GELATIN ACTIVITY.

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I

INTRODUCTION.

Two previous papers¹ showed that the anomalous effect of Mg^{++} ions² on the activity of oxalate diions ($C_2O_4^{--}$) could be counteracted by the addition of salts of Na^+ or K^+ . A similar antagonism was found between SO_4^{--} and Cl^- , as well as between Mg^{++} and SO_4^{--} in their effect on the oxalate diion. No antagonism was found between Na^+ and K^+ .

In this paper we will extend our observations to the effect of single salts and of salt mixtures upon the ionic activity of gelatin.³ It will be shown that, of the three ions Na^+ , K^+ and Mg^{++} , each has its individual effect when present alone with gelatin; and that the effect of a mixture of two or more of these cations is quite different from the effect of one alone.

The experimental data were obtained in the pH range from 6.7 to 7.6 in order to be near the physiological range, the pH of the solutions without salt being 7.367. An accuracy reproducible to 0.001 to 0.003 pH was obtained, although with difficulty.

The ionizable groups of gelatin⁴ are given below together with their degree of ionization at pH 7.367, which was the pH of the gelatin solution without salts in our experiments.

¹ Simms, H. S., *J. Gen. Physiol.*, 1928, xii, 241, 259.

² Simms, H. S., *J. Phys. Chem.*, 1928, xxxii, 1121, 1495.

³ Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 613.

⁴ Simms, H. S., *J. Gen. Physiol.*, 1928, xi, 629.

Acid groups (ionized at pH's higher than pG')	pG' values ⁵	100 α = per cent of ionization	Basic groups (ionized at pH's lower than pG')	pG' values ⁵	100 α = per cent of ionization
Aspartic and glu- tamic acids (R — COOH)	3.5	100	Prearginine ⁴	4.6	0
(Tyrosine R Φ — OH)	9.4	1	Histidine	6.1	5
			Arginine	8.1	84
			Lysine	10.6	100

Arginine is the only group having appreciable amounts of both the ionized and the non-ionized form at this pH (84 and 16 per cent respectively).

To express this in a different way: the change in pH of the gelatin solutions due to addition of salt is an indication of a change in the ionic activity of one or more of the ionic species present. There are only two ionic species of the gelatin present in appreciable quantities;⁶ one in which the arginine group is ionized, the other in which it is non-ionized. The other groups remain essentially the same.

The same statements should apply to any other protein in this range and our results should be typical of proteins in general. At present we have not made observations in other pH ranges.

II.

RESULTS.

A. Solutions Containing Na⁺ and K⁺ Ions.

The effect of Na⁺ (as NaCl) on Na gelatinate may be seen in Figs. 1 and 2A. All the solutions of Na gelatinate contained 1.25 per cent gelatin to which enough NaOH has been added to bring the pH to 7.367 without addition of salt. The pH of this solution is seen to decrease very rapidly with small additions of NaCl; the influence of

⁵ The pG' values (titration indices) represent the mid-points of the buffer ranges due to the respective groups. Simms, H. S., *J. Am. Chem. Soc.*, 1926, xlviii, 1239.

⁶ We neglect the histidine group which is only 5 per cent ionized. That this is permissible is shown by the data on K gelatinate with a salt-free pH of 7.203 where the histidine group is 7 per cent ionized and the arginine group is 89 per cent ionized.

further additions becomes less and less as the concentration of salt increases—but the effect up to above 1.0 molar is to *decrease the pH*.

KCl on the other hand *first decreases* the pH until about 0.01 molar K^+ ion is present. The curve then reverses and further additions of KCl *increase* the pH. This is true whether the KCl is added to Na

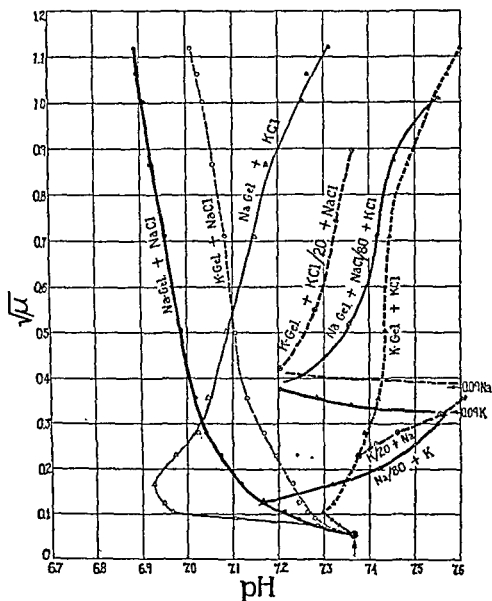


FIG. 1. Effect of NaCl and KCl on the pH of Na gelatin and K gelatin.

We have plotted the square root of the total ionic strength, including that of the Na (or K) gelatin (0.0032 μ). The solid lines represent data with Na gelatin; the broken lines with K gelatin. The two lighter lines are for the effect of one type of chloride on the opposite type of gelatin.

The Z-shaped curves are for solutions containing appreciable amounts of both cations, one being constant and the other varied.

gelatinate or to K gelatinate⁷ (see Figs. 1 or 3A), although in the former case the initial decrease (up to 0.01 M K^+ ion) is much greater. Above 0.01 M K^+ ion concentration the two curves are seen to be parallel.

The Na gelatinate contains 0.0032 M Na^+ ion. In order to observe the effect of more Na^+ ion we then used Na gelatinate containing 0.0125 NaCl (or a total of 0.0157 M Na^+). Addition of KCl then produces quite the opposite effect. Up to about 0.09 M K^+ concentration KCl *increases* the pH; a slight addition of KCl then *decreases* the pH, up to 0.125 M K^+ ; and beyond that further additions *increase* the pH.

The effect of NaCl on K gelatinate is seen to be essentially the same as its effect on Na gelatinate (*i.e.*, a consistent decrease in pH).

The effect of NaCl on K gelatinate containing 0.050 M KCl is almost identical with the reverse process described in the second paragraph above (*i.e.*, first an increase, then a decrease and finally an increase in pH). Furthermore the concentrations of Na^+ ion at the two critical points agree with those of K^+ ion described above, namely, 0.09 M and 0.125 M. It will be seen below that the solutions containing Mg^{++} have critical points nearly identical with these.

B. Solutions Containing Mg^{++} , Na^+ and K^+ Ions.

We now pass to solutions containing Mg^{++} ions in addition to Na^+ or K^+ or both. The curve for the effect of $MgCl_2$ on gelatin is given in both Figs. 2A and 3A. We used Na gelatinate for this experiment and hence have 0.0032 μ of Na^+ ion present. Should we use Mg gelatinate the curve would undoubtedly fall considerably to the left as shown by the extrapolation of the other curves in Figs. 2A or 2B to zero concentration of Na^+ ion.

If we take solutions of Na gelatinate and add a definite amount of $MgCl_2$ plus various amounts of NaCl, the effect up to 0.10 molar Na^+ ion is to *increase* the pH. At 0.10 molar there is a sharp break and a

⁷ The K gelatinate was prepared like the Na gelatinate except that KOH was used instead of NaOH. The pH without salt was 7.203. Hence 0.164 was added to all these values plotted in Figs. 1 and 3 in order that the data might be compared with the Na gelatinate data.

slight further addition of NaCl causes a marked decrease in pH. At 0.15 molar Na^+ ion this effect ceases and further additions of NaCl have very slight effect on the pH. This is demonstrated by the lighter curves in Fig. 2A. In Fig. 2B are plotted the differences between these curves and the curve of NaCl alone (*i.e.*, the observed

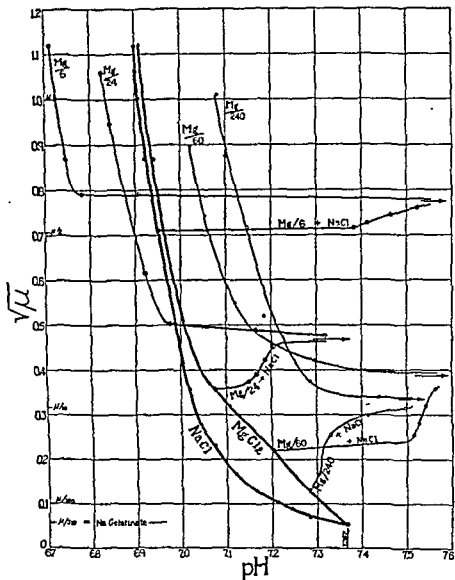


FIG. 2A. Effect of NaCl and MgCl_2 on Na gelatin.

The square root of the total ionic strength is plotted against pH. The two heavy lines represent the effect of NaCl or MgCl_2 alone on Na gelatin. In the latter case the presence of the 0.0032 M Na^+ ion has doubtless affected the curve. Extrapolation of the curves for the mixtures shows that in the absence of Na^+ , MgCl_2 would cause a much more marked depression of the pH.

The lighter curves show the effect of addition of NaCl to Na gelatin containing a fixed amount of MgCl_2 .

pH with MgCl_2 and NaCl , minus the pH with NaCl alone at the same ionic strength). It is obvious that in the presence of MgCl_2 the addition of NaCl produces breaks in the curves at definite concentrations which are independent of the amount of MgCl_2 present (at least from 0.0042 to 0.17 M MgCl_2). Comparing the curves in the higher concentrations of NaCl , it is evident that in this range, addition of MgCl_2 lowers the pH.

It might be added that the effect on pH is not due to the sequence of addition of the salts. Three solutions were prepared with the same amounts of NaCl , MgCl_2 and Na gelatinate, but the components were

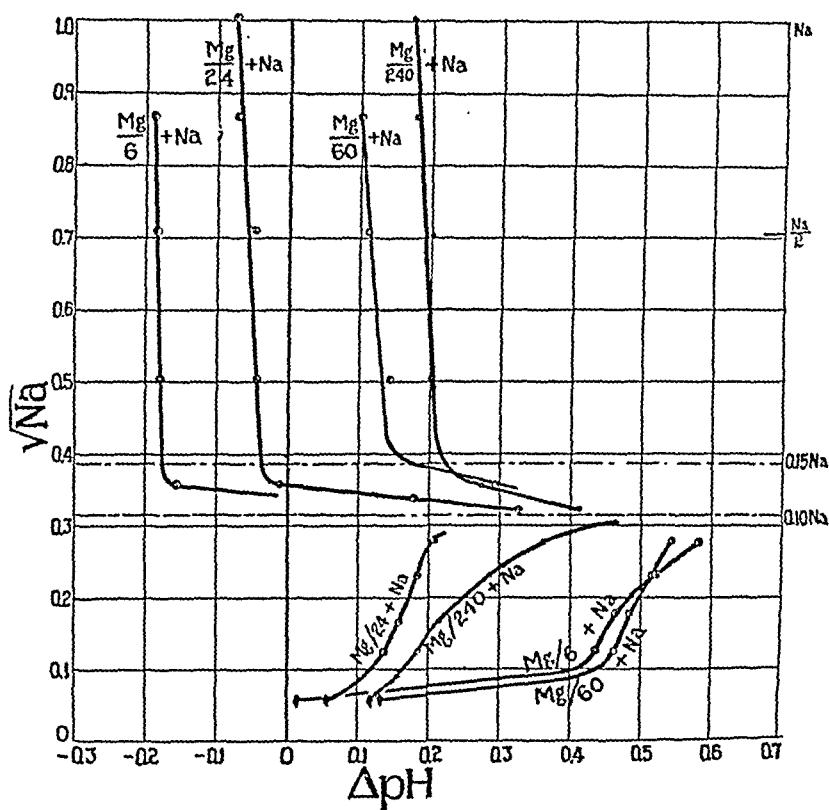


FIG. 2B. Effect of $\text{Na}^+ - \text{Mg}^{++}$ mixtures relative to that of NaCl alone.

The values for ΔpH represent the observed pH values minus the pH with the same ionic strength of NaCl . These are plotted against the square root of the Na^+ concentration. (The Mg^{++} is not included.) It is seen that the "breaks" are independent of the amount of MgCl_2 .

mixed in different order in each case. All three had essentially the same pH.

The effect of KCl on gelatin containing MgCl_2 is almost identical up to 0.12 M K^+ ion with the effect of NaCl (compare Fig. 3A with

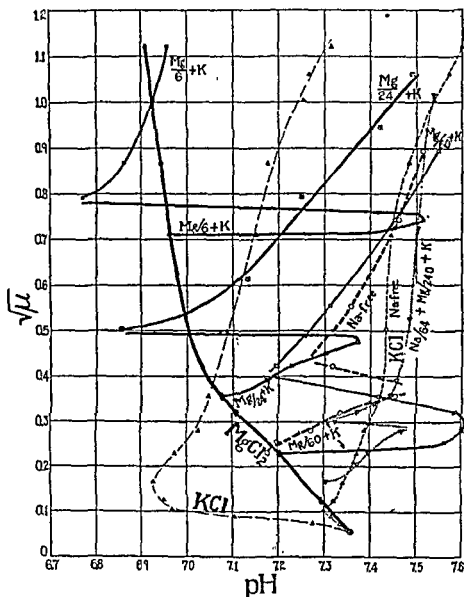


FIG. 3A. Effect of KCl and MgCl_2 on the pH of Na gelatin and K gelatin. Also the effect of a mixture of Mg, Na and K.

Curves are given for the effects of MgCl_2 alone on Na gelatin, KCl alone on Na gelatin and KCl alone on K gelatin (indicated "Na-free").

The effects of KCl on Na gelatin containing fixed amounts of MgCl_2 are represented by solid Z-shaped curves. Using K gelatin gives the Z-shaped curve marked "Na-free" (dash line).

The effect of appreciable amounts of all three cations is given by the dotted line.

2A and Fig. 3B with 2B). Above the point of the second break (0.12 M K^+ ion) the curves in the case of KCl go to the right (higher pH) which is not surprising since KCl alone in higher concentrations causes an increase in pH.

In Fig. 3B we have plotted the difference between the curves with $MgCl_2$ plus KCl and the curve with KCl alone. This gives the effect of the mixture, and eliminates the effect of KCl alone. The dash line gives the observations on the addition of KCl to K gelatinate containing

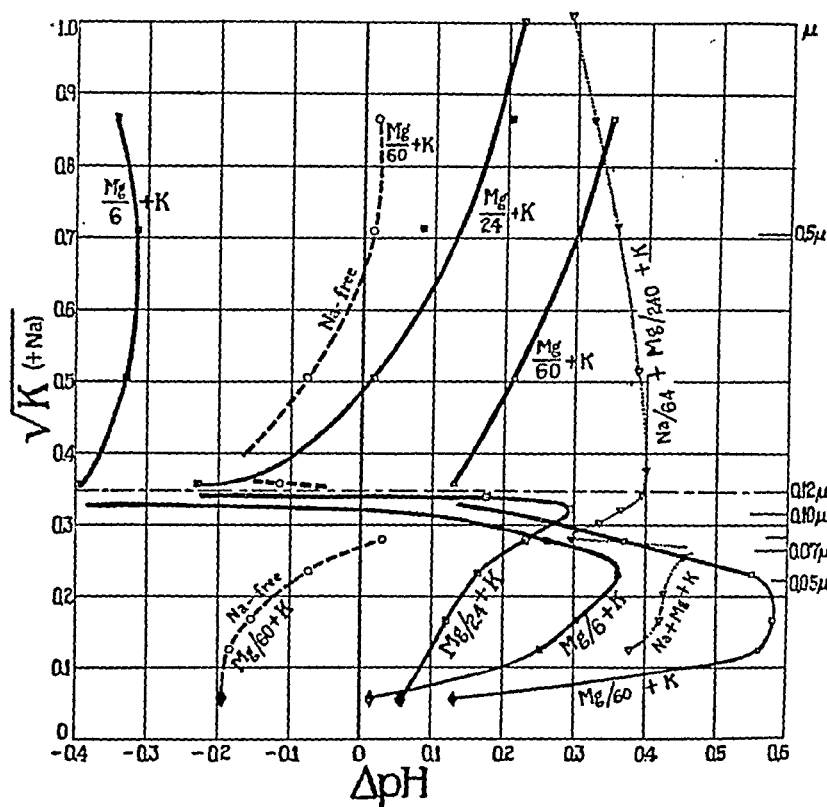


FIG. 3B. Effect of $K^+ - Mg^{++}$ mixtures or a $K^+ - Na^+ - Mg^{++}$ mixture, relative to that of KCl alone.

The values of ΔpH equal the observed pH values minus the pH with the same ionic strength of KCl (on Na gelatinate or K gelatinate as the case may be). These are plotted against the square root of K^+ plus Na^+ concentration. (The Mg^{++} is not included.) It is seen that the "breaks" are independent of the amount of $MgCl_2$. (Cf. Fig. 2B.)

MgCl_2 . Since no Na^+ was present the effect of this ion does not enter in, but it will be seen that this curve is essentially like those in Fig. 2B (with Na gelatinate and NaCl).

The *heavy* lines in Fig. 3B (also 3A) represent data on Na gelatinate and hence contain 0.0032 M Na^+ ion. These are seen to be essentially like that with K gelatinate. The *dotted* line represents similar solutions with an added amount of NaCl, raising the Na^+ ion concentration to 0.0157 M. This curve is essentially like the others except that the critical points come at slightly lower concentration of $[\text{Na}^+ + \text{K}^+]$, namely at 0.07 M $[\text{Na}^+ + \text{K}^+]$ and at 0.08 M $[\text{Na}^+ + \text{K}^+]$. There is a third break at 0.12 M, but not a sharp one.

III.

DISCUSSION.

The above observations, while incomplete, may throw light on some of the physiological anomalies, such as the antagonistic effects of cations; the failure of Na^+ alone or K^+ alone to supplant a mixture of the two in physiological systems; and the unequal distribution of ions between the various physiological fluids, secretions and tissues in a given organism.

The essential qualitative generalizations to be made from the above observations are:

1. Mixtures of NaCl and KCl behave differently from either salt alone, in their effect on gelatin activity.
2. Mixtures of MgCl_2 and NaCl (or KCl) behave differently from these salts alone, the effect being slightly different if KCl is used in place of NaCl.
3. Mixtures containing all three cations, Mg^{++} , Na^+ and K^+ in appreciable quantities, behave similarly to Mg^{++} plus NaCl or KCl, but the curve is complicated by three breaks instead of two.

IV.

THEORY.

Before attempting to explain the above phenomena, we may remind the reader that previous observations^{2,8} have shown a tendency of cations to affect the ionic activity of simple amines and ampholytes, in

* Simms, H. S., *J. Phys. Chem.*, 1929, xxxiii, in press.

a manner not in agreement with the Debye-Hückel theory and corresponding to the complex salts observed by Pfeiffer and collaborators.⁹ These deviations with gelatin also agree with the combinations of ions with proteins found by Northrop and Kunitz¹⁰ in a different manner.

It would seem that the various cations have an affinity for certain portions of the protein molecule but it is at first difficult to understand how two cations tending individually to produce one effect can produce the opposite effect in a mixture.

Let us consider the gelatin in this pH range to consist of two ionic species. The first, which we will call *A*, has its arginine group ionized, and predominates at lower pH values. The second, *B*, has its arginine group non-ionized, and predominates at higher pH.

Let us assume, for example, that in a given concentration, the tendency for Na^+ ion to combine with *A* is such as to produce negligible effect on its activity, while Na^+ reduces the activity of *B* to 0.9; then its effect on the arginine dissociation index (8.1) of gelatin would be represented by the logarithm of the ratio of γ_B/γ_A . The change in pH would in this range equal the change in pK; hence:

$$\Delta\text{pH} = \Delta \text{pK}' = \log \gamma_B/\gamma_A$$

or

$$\Delta\text{pH} = \log 0.9/1.0 = -0.05.$$

Let us assume furthermore that a given concentration of Mg^{++} ion reduces the activity of *A* to 0.5 and the activity of *B* to 0.3; then

$$\Delta\text{pH} = \log 0.3/0.5 = -0.22.$$

Thus Mg^{++} produces an effect in the same direction as Na^+ .

Let us assume thirdly that in a mixture of the same amounts of Na^+ and Mg^{++} ions the species *A* is affected principally by Mg^{++} ion ($\gamma_A = 0.5$) and that *B* is affected principally by Na^+ ion ($\gamma_B = 0.9$). It is then obvious that the effect on the pH will be in the *opposite direction* from the effect with Na^+ or Mg^{++} alone, since

⁹ Pfeiffer, P., and Wittha, F., *Ber. chem. Ges.*, 1915, xlviii, 1289. Pfeiffer, P., Würgler, J., and Wittha, F., *Ber. chem. Ges.*, 1915, xlviii, 1938; *Z. physik. Chem.*, 1924, cxxxiii, 22; cxxxiv, 180; cxxxv, 16; cxliii, 265.

¹⁰ Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1928, xi, 481.

$$\Delta pH = \log 0.9/0.5 = +0.25.$$

The first two of the above assumptions are legitimate and reasonable. The third assumption is made to agree with the observations. If it is correct it means that in a mixture of Na^+ and Mg^{++} these ions do not combine with the two ionic species of gelatin in the same ratio that they do when present alone, but that Na^+ combines more with one ionic species and Mg^{++} more with the other. (Although this might be accounted for with Na^+ and Mg^{++} at low concentrations on the basis of the charge, such an explanation would fail in the case of Na^+ and K^+ .)

V.

EXPERIMENTAL.

2.50 per cent gelatin solutions were made up in 250 cc. amounts, and containing 16 cc. 0.100 M NaOH , or KOH . These were used as mother solutions in all measurements.

For each observation 5.00 cc. of mother solution was placed in a 10 cc. volumetric flask; the requisite amounts of the various salts were added, and the solution made up to 10.00 cc. (1.25 per cent gelatin). The salts were added volumetrically from stock solution in three concentrations (0.025μ , 0.25μ and 2.50μ).

The pH of each solution was measured at 25.0°C . in a water-jacketed hydrogen electrode of the bubbling type.¹¹

The accuracy of the measurements needs mentioning. It is difficult to get accurate readings on protein solutions in this range, but by frequent and careful plating of the electrodes, and by passing the hydrogen over red hot copper, it was possible to make readings reproducible in general to 0.0001 volt (0.001 pH). As an illustration, fresh mother solution with NaOH was made up six times, and each time the pH without salt was exactly 7.367. (Two solutions with KOH had the same pH of 7.302.)

The readings with salts were discarded if at least two consecutive readings out of three readings on each solution did not agree within 0.0002 volt (0.003 pH). Furthermore all data which from the nature of the curve seemed doubtful were repeated, and were discarded if they were not corroborated. New solutions were then observed with new electrodes.

The data are given in Tables I to XI.

¹¹ Simms, H. S., *J. Am. Chem. Soc.*, 1923, xlv, 2503.

TABLE I.
Effect of NaCl on Na Gelatinate.

C_{NaCl}	pH	C_{NaCl}	pH	C_{NaCl}	pH
0	7.367	0.025	7.120	0.500	6.954
0.0025	7.284	.050	7.073	.750	6.921
.0050	7.243	.075	7.041	1.000	6.907
.0075	7.213	.125	7.018	1.125	6.903
.0125	7.164	.250	6.987	1.250	6.900

TABLE II.
Effect of KCl on Na Gelatinate.

C_{KCl}	pH	C_{KCl}	pH	C_{KCl}	pH
0	7.367	0.025	6.927	0.500	7.147
0.0025	7.283	.050	6.973	.750	7.176
.0050	7.103	.075	7.025	1.000	7.249
.0075	6.968	.125	7.046	1.125	7.267
.0125	6.949	.250	7.086	1.250	7.311

TABLE III.
Effect of KCl on K Gelatinate.

0.164 is added to the following pH values to give those plotted in Figs. 1 and 3A.

C_{KCl}	pH	C_{KCl}	pH	C_{KCl}	pH
0	7.203	0.025	7.178	0.500	7.281
0.0025	7.169	.050	7.209	.750	7.318
.0050	7.152	.075	7.225	1.000	7.374
.0075	7.135	.125	7.256	1.125	7.406
.0125	7.156	.250	7.269	1.250	7.436

TABLE IV.
Effect of NaCl on K Gelatinate.

0.164 is added to these pH values to give those plotted in Fig. 1.

C_{NaCl}	pH	C_{NaCl}	pH	C_{NaCl}	pH
0	7.203	0.025	7.068	0.500	6.921
0.0025	7.137	.050	7.032	.750	6.895
.0050	7.115	.075	7.005	1.000	6.873
.0075	7.099	.125	6.968	1.125	6.863
.0125	7.081	.250	6.941	1.250	6.846

TABLE V.

Effect of KCl on Na Gelatinate Containing 0.0125 M NaCl.

(pH without salt = 7.367)

 μ for each solution equals $C_{KCl} + 0.0157$.

C_{KCl}	pH	C_{KCl}	pH
0	7.164	0.112	7.281
0.0125	7.318	.125	7.203
.025	7.419	.250	7.354
.050	7.499	.500	7.416
.075	7.549	.750	7.453
.087	7.563	1.000	7.548
.100	7.359		

TABLE VI.

Effect of NaCl on K gelatinate Containing 0.050 M KCl.

(pH without salt = 7.203)

0.164 is added to these pH values to give those plotted in Fig. 1.

 μ for each solution equals $C_{NaCl} + 0.0532$.

C_{NaCl}	pH	C_{NaCl}	pH
0	7.213	0.125	7.042
0.0125	7.249	.250	7.117
.0250	7.298	.500	7.164
.0500	7.393	.750	7.198
.0750	7.446		

TABLE VII.

Effect of $MgCl_2$ on Na Gelatinate.

C_{MgCl_2}	pH	C_{MgCl_2}	pH	C_{MgCl_2}	pH
0	7.367	0.100	7.108	0.750	6.942
0.0125	7.293	.125	7.074	1.250	6.910
.050	7.203	.500	6.963		

TABLE VIII.

Effect of MgCl₂ Plus NaCl on Na Gelatinate Containing MgCl₂.
(pH without salt = 7.367)

A. 0.00416 M MgCl ₂ plus various amounts of NaCl ($\mu = C_{\text{NaCl}} + 0.0157$)					
C_{NaCl}	pH	ΔpH^*	C_{NaCl}	pH	ΔpH^*
0	7.285	0.117	0.100	7.438	0.411
0.0125	7.305	.177	.125	7.287	.274
.0250	7.306	.216	.250	7.184	.201
.0500	7.331	.287	.500	7.149	.200
.0750	7.414	.373	.750	7.101	.177
.0875	7.497	.463	1.000	7.083	.174
B. 0.0167 M MgCl ₂ plus various amounts of NaCl ($\mu = C_{\text{NaCl}} + 0.0532$)					
0	7.203	0.131	0.125	7.296	0.294
0.0125	7.516	.461	.250	7.120	.141
.025	7.526	.485	.500	7.054	.110
.050	7.543	.518	.750	7.021	.100
.075	7.563	.545			
C. 0.0416 M MgCl ₂ plus various amounts of NaCl ($\mu = C_{\text{NaCl}} + 0.128$)					
0	7.074	0.057	0.112	7.167	0.178
0.0125	7.151	.138	.125	6.977	-.010
.025	7.169	.159	.250	6.922	-.045
.050	7.186	.184	.500	6.905	-.048
.075	7.205	.209	.750	6.845	-.072
.100	7.319	.328	1.000	6.827	-.076
D. 0.167 M MgCl ₂ plus various amounts of NaCl ($\mu = C_{\text{NaCl}} + 0.503$)					
0	6.963	0.013	0.125	6.780	-0.156
0.0125	7.384	.436	.250	6.746	-.180
.025	7.413	.467	.500	6.724	-.185
.050	7.465	.521	.750	6.711	-.190
.075	7.523	.583	1.000		

* The values of ΔpH in this table equal the above pH value minus the pH of the same ionic strength of Na gelatinate plus NaCl (Fig. 1 or 2A).

TABLE IX.

Effect of KCl on Na Gelatinate Containing $MgCl_2$.
(pH without salt = 7.367)

A. 0.0167 M $MgCl_2$ plus various amounts of KCl ($\mu = C_{KCl} + 0.0532$)					
C_{KCl}	pH	ΔpH^*	C_{KCl}	pH	ΔpH^*
0	7.203	0.131	0.125	7.193	0.130
0.0125	7.563	.560	.250	7.311	.212
.025	7.600	.580	.500	7.452	.300
.050	7.585	.550	.750	7.548	.347
.075	7.416	.370			
B. 0.0416 M $MgCl_2$ plus various amounts of KCl ($\mu = C_{KCl} + 0.128$)					
0	7.074	0.057	0.125	6.856	-0.230
0.025	7.174	.120	.250	7.133	+.016
.050	7.227	.164	.500	7.250	.083
.075	7.301	.230	.750	7.421	.205
.100	7.371	.292	1.000	7.491	.220
.112	7.258	.176			
C. 0.167 M $MgCl_2$ plus various amounts of KCl ($\mu = C_{KCl} + 0.503$)					
0	6.963	0.013	0.125	6.773	-0.394
0.0125	7.398	.252	.250	6.861	-.330
.025	7.450	.302	.500	6.927	-.316
.050	7.514	.360	.750	6.956	-.344
.075	7.416	.260			

* The values of ΔpH in this table equal the above pH values minus the pH of the same ionic strength of Na gelatinate plus KCl (Fig. 1).

TABLE X.

Effect of KCl on Na Gelatinate Containing 0.0125 M NaCl plus 0.00417 M MgCl₂.
 (pH without salt = 7.367)
 μ for each solution equals $C_{\text{KCl}} + 0.0282$.

C_{KCl}	pH	ΔpH^*	C_{KCl}	pH	ΔpH^*
0	7.305	0.378	0.100	7.441	0.394
0.0125	7.372	.420	.125	7.457	.400
.0250	7.398	.425	.250	7.484	.387
.0500	7.470	.452	.500	7.504	.354
.0625	7.323	.295	.750	7.514	.320
.0750	7.370	.334	1.000	7.538	.288
.0875	7.406	.363			

* The values of ΔpH in this table equal the above pH values minus the pH of the same ionic strength of Na gelatinate plus KCl (Fig. 1 or 3A).

TABLE XI.

Effect of KCl on K Gelatinate Containing 0.0167 M MgCl₂.
 (pH without salt = 7.203)

0.164 is added to these pH values to give those plotted in Figs. 3A and 3B.
 μ for each solution equals $C_{\text{KCl}} + 0.0532$.

C_{KCl}	pH	ΔpH^*	C_{KCl}	pH	ΔpH^*
0	7.014	-0.194	0.125	7.154	-0.113
0.0125	7.035	-.183	.250	7.193	-.078
.0250	7.073	-.153	.500	7.298	+.012
.0500	7.171	-.073	.750	7.350	+.020
.0750	7.289	+.030			

* The values of ΔpH in this table equal the above pH values minus the pH of the same ionic strength of K gelatinate plus KCl (Fig. 1 or 3A). It will be noted that these solutions are free from Na.

VI.

SUMMARY.

1.25 per cent gelatin solutions containing enough NaOH to bring them to pH 7.367 (or KOH to pH 7.203) were made up with various concentrations of NaCl, KCl and $MgCl_2$, alone and in mixtures, up to molar ionic strength. The effects of these salts on the pH were observed.

$MgCl_2$ and NaCl alone *lower* the pH of the Na gelatinate or the K gelatinate, in all amounts of these salts.

KCl first *lowers* the pH (up to 0.01 M K^+), then *raises* the pH.

Mixtures of NaCl and KCl (up to 0.09 M of the salt whose concentration is varied) *raise* the pH; then (up to 0.125 M Na^+ or K^+) *lower* the pH; and finally (above 0.125 M) behave like KCl alone.

Mixtures of $MgCl_2$ and NaCl *raise* the pH up to 0.10 M Na^+ , and *lower* it up to 0.15 M Na^+ *regardless of the amount of $MgCl_2$* . Higher concentrations of NaCl have little effect, but the pH in this range of NaCl concentration is lowered with increase of $MgCl_2$.

Mixtures of $MgCl_2$ and KCl behave as above described (for $MgCl_2$ and NaCl) and the addition of NaCl *plus* KCl to gelatin containing $MgCl_2$ produces essentially the same effect as the addition of either alone, except that the first two breaks in this curve come at 0.07 M and 0.08 M [$Na^+ + K^+$] and there is a third break at 0.12 M.

In this pH range the free groups of the dicarboxylic acids and of lysine are essentially all ionized and the prearginine and histidine groups are essentially all non-ionized. The arginine group is about 84 per cent ionized. Hence we are studying a solution with two ionic species in equilibrium, one with the arginine group ionized, and one with it non-ionized.

It is shown that the effect of each salt alone depends upon the effect of the cation on the activity of these two species due to combination. The anomalous effects of cation mixtures may be qualitatively accounted for if one or both of these species fail to combine with the cations in a mixture in proportion to the relative combination in solutions of each cation alone.

Special precautions were taken to ensure accuracy in the pH measurements. The mother solutions gave identical readings to 0.001 pH and the readings with salts were discarded when not reproducible to 0.003 pH. All doubtful data were discarded.

CHEMICAL AND PHYSICAL CHANGES IN GELATIN SOLUTIONS DURING HYDROLYSIS.

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The initial stages of the hydrolysis of gelatin solutions by either enzymes or acids are accompanied by a very marked decrease in the viscosity of the solution. In fact the viscosity may change 20 or 30 per cent before any chemical change can be noted unless very sensitive methods such as conductivity are used. It is not surprising, therefore, that the "liquefying" action of enzymes has been at times considered as purely physical and distinct from their hydrolytic action. As Waldschmidt-Leitz (1) has emphasized, however, all our knowledge of protein hydrolysis goes to show that the reaction is purely chemical and consists in the rupture of a peptide linking. Nevertheless, it would not be expected that such a reaction would cause any marked change in the viscosity, since viscosity, according to Einstein (2), is a function merely of the volume of the solute, and two small molecules should occupy nearly the same volume when separate as when they are combined. In many cases, such as the hydrolysis of sugar or of dipeptides or even of proteins having a low viscosity, this is true and the hydrolysis is accompanied by only slight changes in viscosity. In the case of gelatin, however, the change in viscosity is relatively enormous and it is evident that there is some secondary mechanism by means of which a very small chemical change gives rise to a very large physical one. The explanation of the viscosity of gelatin solutions assumed by Loeb (3) and extended by Kunitz (4) furnishes the required mechanism. According to this hypothesis a gelatin solution contains particles or micelles consisting probably of an insoluble material, enclosing and surrounded by a solution of soluble material. At about 10 per cent total concentra-

tion the concentration of the gelatin is the same inside and outside of the particles. Below 10 per cent the gelatin is more concentrated inside than outside the micells. When acid or alkali is added a Donnan equilibrium is set up between these micells and the surrounding solution and they swell, thereby increasing the viscosity of the solution. If salt is now added the Donnan effect is lessened, the micells shrink and the viscosity decreases. The same effect could be obtained by adding to the solution some material which could not penetrate the micells. When the total concentration is 10 per cent or higher the concentration of gelatin in the micells is the same as that of the outside solution and adding acid therefore has no effect. Let us assume now that the action of enzymes or of acid destroys the micells either by hydrolyzing the insoluble network or the internal solute. Both effects probably occur. In either event the result will be a marked decrease in the volume of the micells. A very slight chemical action will thereby cause a very large physical change. Under certain conditions there will be a further secondary effect. If the micells are swollen due to the presence of acid the material liberated from the destroyed micells will depress the swelling of the remaining intact ones just as would the addition of a salt. It would be expected therefore that the change in viscosity, or better, in the volume of solute, for a given amount of chemical change should be greatest when the viscosity is due to swelling of the micells, as in acid, and should be less at the isoelectric point, or when salt had been added, or in concentrated gelatin solutions.

In order to test this assumption gelatin was hydrolyzed by pepsin and the increase in formol titration and decrease in viscosity determined simultaneously.

The gelatin was prepared as previously described (5). The viscosity was determined by du Noüy's (6) torsion viscometer and the increase in the formol titration determined by the method described in a preceding paper (7). The experiments were carried out in a constant temperature bath at 37°C.

The results of the experiments are shown graphically in Figs. 1, 2 and 3, in which the decrease in viscosity has been plotted against the corresponding increase in formol titration. Fig. 1 gives the result of an experiment with 1 per cent gelatin at various pH values. The

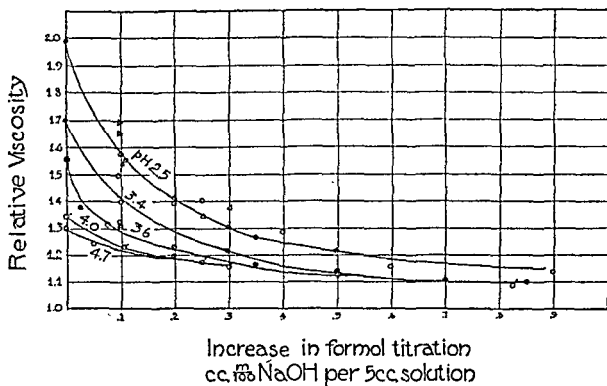


FIG. 1. Decrease in viscosity of 1 per cent gelatin solutions at various pH values, as a function of the increase in formol titration.

TABLE I.

Per Cent Change in Viscosity or Volume of Gelatin Solutions under Various Conditions Caused by 5 Per Cent Increase in Formol Titration.

Concentration of gelatin..	1 per cent							8.5 per cent		
pH.....	4.7	4.0	3.7	3.4	3.0 or 2.5	3.0	3.0	4.7	4.0	3.5 or 3.0
Salt concentration..	0	0	0	0	0	0.1M	0.4M	0	0	
Original viscosity (H ₂ O = 1.0).....	1.30	1.35	1.60	1.70	2.0	1.45	1.35	13.0	14.0	15.0
Final viscosity.....	1.19	1.19	1.23	1.29	1.4	1.26	1.21	6.0	6.8	7.0
Per cent decrease in viscosity actual. . .	8	12	23	24	30	14	10	54	51	53
(corrected for H ₂ O).....	37	46	62	59	60	42	40	59	55	60
Original volume gela- tin cc. per 100 cc. solution.....	5.8	6.5	10.0	11.3	14.5	8.0	6.5	44.7	45.6	46.5
Final volume gelatin.	3.9	3.9	4.6	5.6	7.3	5.1	4.3	33.4	35.4	35.8
Per cent decrease in volume.....	33	40	54	50	50	36	34	26	23	23

experiment shows that the greater the initial viscosity the greater the change in viscosity for a given chemical change. This is in accord with the mechanism outlined above since the more swollen the micells are, the greater change in volume and hence in viscosity will occur when a micell is ruptured. If the results are expressed as per cent change in viscosity for a 5 per cent increase in formol titration (Table I) it is found that the percentage change for a constant chemical effect is also greater the higher the viscosity. This is true whether

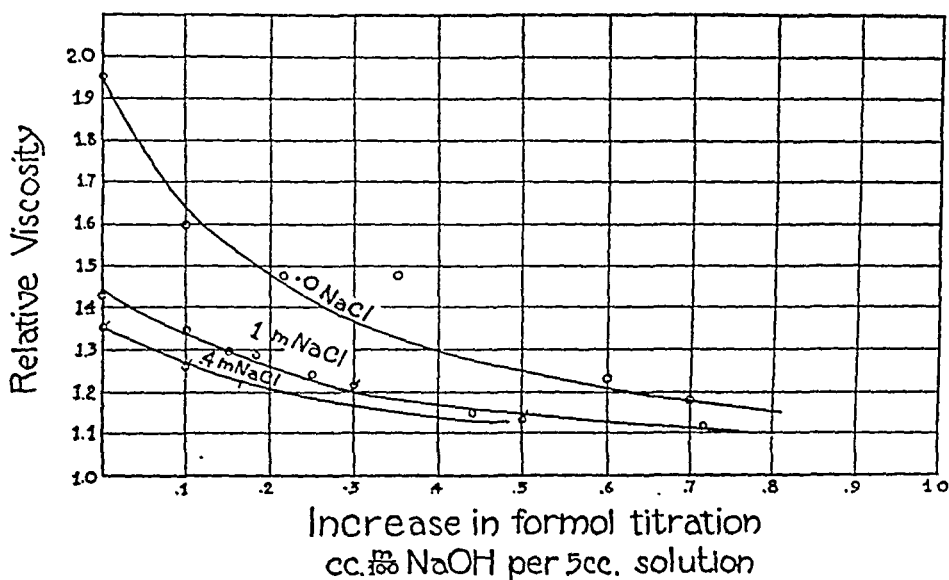


FIG. 2. Decrease in viscosity of 1 per cent gelatin at pH 3.0 with increasing salt concentration, as a function of the increase in formol titration.

the actual viscosity or the viscosity corrected for that of water is used. If, instead of the viscosity, the change in volume according to Kunitz's (8) equation corresponding to this viscosity change is used, the result is the same except that the percentage differences are not so marked. The fact that the percentage change in viscosity is greater the greater the initial viscosity is due to the secondary effect mentioned above. If the action consisted simply in the rupture of the micells the percentage loss of volume of the micells due to the rupture of the same number should be the same, and should be independent of the size of the micells. This is not the case, since when

the micells are swollen and the viscosity therefore high, as at pH 3.0, the change in viscosity is greater for a 5 per cent increase in carboxyl groups (formol titration) than at the isoelectric point where the micells are less swollen. This effect is probably due, as stated above, to the influence of the material liberated by the rupture of a micell on the other micells. When the micelles are swollen due to a Donnan equilibrium the presence of more soluble material will result in

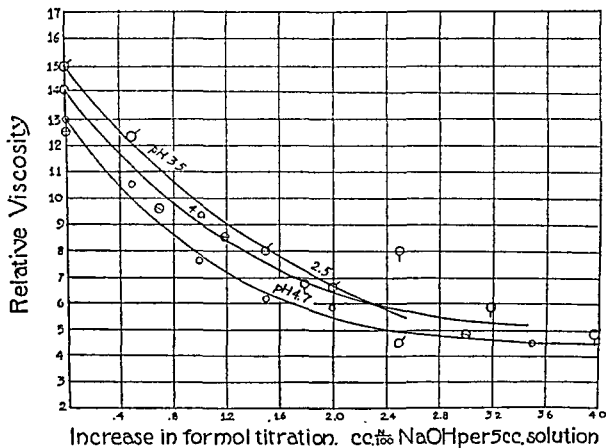


FIG. 3. Decrease in viscosity of 8 per cent gelatin at various pH values, as a function of the increase in formol titration.

a decrease in the osmotic pressure due to this equilibrium and the micells will therefore shrink. At the isoelectric point, where there is no Donnan equilibrium and the micells are less swollen, the material liberated from the ruptured micells will have little or no effect on the intact ones. It could also be assumed that, when the micells are highly swollen, less chemical hydrolysis is necessary to rupture them. Probably both mechanisms are at work.

If the swelling of the micells at pH 3.0 is repressed by the addition of salt evidently the change in viscosity for the same percentage change in the number of carboxyl groups should be the same as at the isoelectric point, and this is the result as shown in Fig. 2 and Table I.

At a concentration of gelatin of about 8 per cent the effect of adding acid on the viscosity practically disappears and this has been explained by Kunitz (4) on the assumption that the concentration of gelatin is the same inside and outside of the micells. Under these conditions, therefore, the percentage decrease in volume of solute for an equal percentage increase in carboxyl groups (rupture of an equal per cent of the number of micells) should be the same at all pH and equal to the percentage change found in isoelectric gelatin. This is the case, as shown in Fig. 3 and the last three columns of Table I. It will be noted in the table that the change in the actual viscosity is greater in the concentrated gelatin than in the 1 per cent but that when the viscosity readings are transformed to volume changes this difference disappears.

SUMMARY.

1. The change in viscosity and the corresponding increase in the carboxyl groups, as determined by the formol titration, has been determined in gelatin solutions during the progress of hydrolysis by pepsin.

2. Very marked changes in viscosity are found to result from very slight chemical changes. If the viscosity is increased by the addition of acid a greater change in viscosity (volume of solute) is caused by the same percentage change in the number of carboxyl groups. The percentage change in the volume of solute, caused by the same percentage increase in the number of carboxyl groups, is independent of the concentration of gelatin.

3. These results are in agreement with the idea that the high viscosity of gelatin solutions is due to the presence of swollen micells, since a slight chemical hydrolysis may be sufficient to rupture a micella and so cause a very large change in viscosity.

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THE SWELLING OF GELATIN AND THE VOLUME OF SURROUNDING SOLUTION.

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The theory of the swelling of gelatin in acid as developed by Procter and Wilson and by Loeb (1) is based on the Donnan equilibrium and assumes that the swelling is due to the osmotic pressure caused by the unequal distribution of the ions of the acid inside and outside of the gelatin. At equilibrium this osmotic pressure for strong monovalent acids is given by the expression

$$(1) \quad 2 Y + Z - 2 X$$

X = concentration acid in supernatant liquid.
 Y = " free acid in gelatin.
 Z = " non-diffusible ion = concentration Cl^- equivalent to ionized protein.

It follows that with pure gelatin at equilibrium in acid the swelling depends only on the *concentration* of acid outside the gelatin and is independent of the *volume* of the acid. The equilibrium concentration of acid and hence the swelling will depend, however, on the quantity as well as the concentration of the acid originally taken. The quantitative statement of this effect is complicated and has been discussed by Hitchcock (2). Qualitatively, however, the result may be simply predicted. When the swelling of gelatin in HCl is plotted against the pH of the acid there is found a maximum at about pH 3.0. If increasing volume of acid solution having a pH of 3.0 or higher is added to a series of constant amounts of gelatin, therefore, the pH of the solution at equilibrium will approach the initial pH of the acid as more acid is added. The swelling will therefore increase with increasing amount of acid solution and finally reach a maximum constant value corresponding to the pH of the pure acid. This will be true as soon as the amount

of acid which combined with the gelatin is negligible compared to the total amount of acid added. If acid of greater strength than pH 3.0 is used the pH will decrease again as the amount of acid solution added is increased and will again become constant when the amount of solution added is so large that there is no change in pH on addition of the gelatin. The swelling will now increase with the volume of solution, reach a maximum (at pH 3.0) and then decrease to a constant value. Since it is necessary for equilibrium that there be some excess solution, *i.e.*, that sufficient solution is used so that some remains after the gelatin is swollen, it would be possible with very strong acid to bring the gelatin to a pH less than 3.0 before there is any excess solution. In this case, after enough solution has been added to furnish an excess of liquid the swelling will decrease as the amount of acid solution added increases. If the swelling obtained in the different series is plotted against the volume of the original acid solution used, therefore, the swelling will increase, decrease or pass through a maximum with increasing volume of solution, depending on the original concentration of the acid solution. Such results have been recorded in the literature (3). If, however, the swelling values from these experiments are plotted against the pH of the solution (either of the gelatin or supernatant solution) at equilibrium, then the swelling should be independent of the volume of solution. According to Küntzel (3), however, this is not the case. Küntzel found that even when the swelling was plotted against the pH of the solution at equilibrium, the swelling increased with increasing volume of the solution. This result is contrary to that expected from the theory for the case of pure acid and gelatin, but is the expected result if the *gelatin contained neutral salts*. The swelling is depressed by neutral salts and hence the less the volume of acid added the greater the depressing effect of the salt. The swelling even at constant pH will therefore increase with the volume of acid added until the salt is so dilute as to have no effect. This is the result obtained by Küntzel (3). Küntzel used gelatin without previous purification and hence salts were present. In order to be sure of this explanation, however, experiments similar to Küntzel's were done with ordinary gelatin and also with isoelectric gelatin. The results with isoelectric gelatin are given in Fig. 1 in which the swelling at equilibrium has been plotted against the volume

of acids of different concentration added. As predicted, the swelling with dilute acids increases with the quantity of acid. The swelling in $M/50$ acid passes through a maximum while stronger acid gives decreasing swelling with increasing volume. When these curves are plotted against pH at equilibrium (either of the gelatin or outside

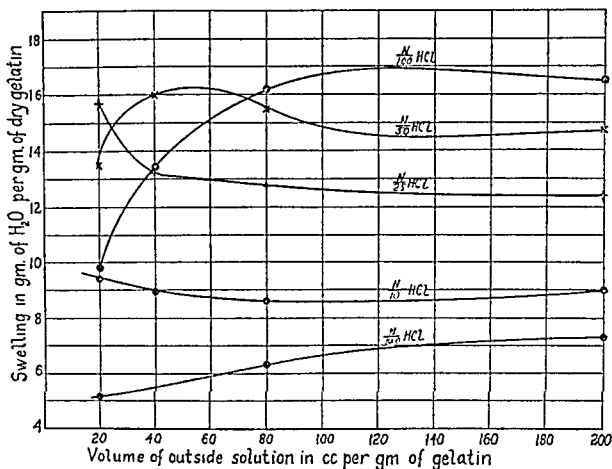


FIG. 1. Swelling of gelatin as determined by the volume of acid of various concentrations.

solution) they coincide as shown in Fig. 2, giving the usual pH swelling curve for gelatin in HCl. When the experiment is performed with unpurified gelatin the results shown in Fig. 3 are obtained which agree with those of Kuntzel. As stated above the increase in swelling with increased volume in this case is due to the decreased salt concentration. It may be mentioned that the absolute amount of swelling cannot be compared in experiments done with different samples of gelatin since this property varies markedly in different preparations.

Experimental Procedure.

Cooper's powdered gelatin was used. The isoelectric gelatin was prepared as described previously (4). The experiments were carried out in a water bath at 5°C. 200 cc. of acid was placed in a series of 500 cc. Erlenmeyer flasks and brought to the temperature of the bath. The flasks were equipped with mechanical stirrers which rotated at approximately the same speed. The gelatin was then

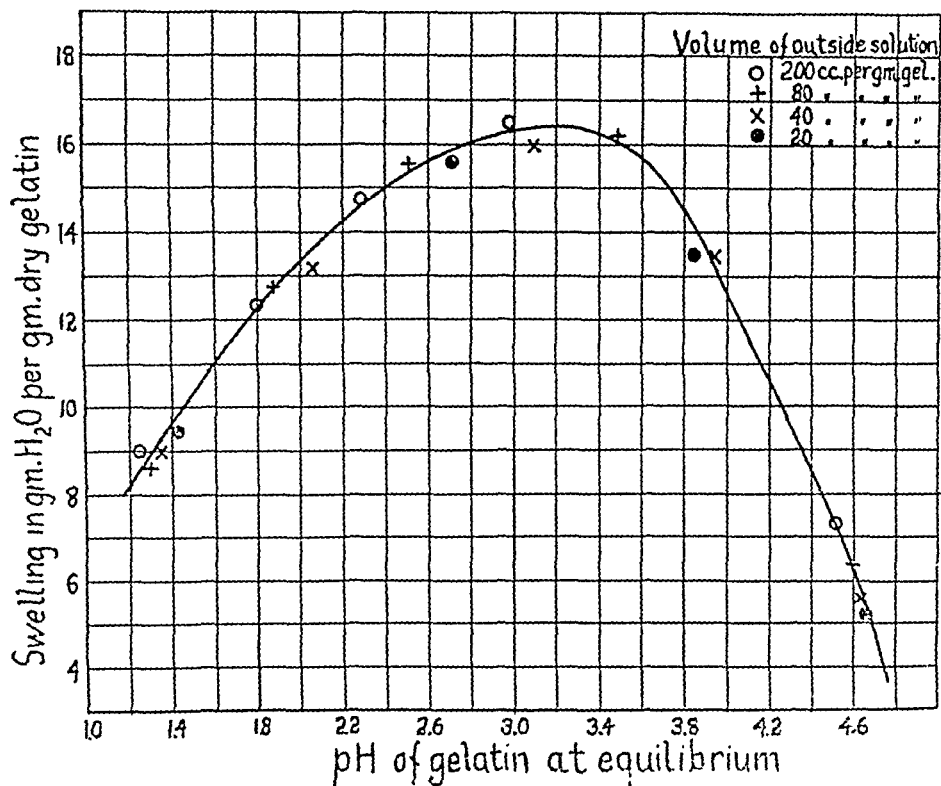


FIG. 2. Swelling of purified gelatin with increasing volume of acid solution as a function of the pH of the gelatin.

added and the suspensions stirred for 5 hours. Preliminary experiments showed that equilibrium was reached in about 2 hours. The gelatin was then filtered off with suction through coarse filter paper and the concentration of the swollen gelatin determined by drying a weighed sample to constant weight at 100°C. Dry weight determinations were also made on the supernatant solution. No appreciable amount of gelatin was found in the supernatant. The pH of the melted gelatin and of the outside solution were determined electrometrically at 30°C.

It may be pointed out that a number of sources of error must be guarded against in swelling experiments of this type. If the temperature is much above 5° there will be some solution of the gelatin and this will increase with the acidity and volume of the solution. The final volume depends to a slight extent on the time required for the swelling to take place, since a true equilibrium is never reached but the mass of gelatin continues slowly to swell indefinitely. If precautions

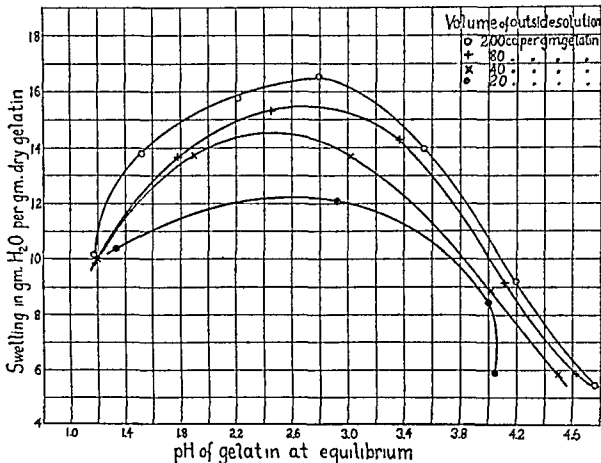


FIG. 3. Swelling of gelatin containing electrolytes with increasing volume of acid solution as a function of the pH of the gelatin.

for rapid stirring are not taken, therefore, the gelatin in a large volume of acid will swell more slowly than that in a small volume and the figure obtained for the equilibrium value will be slightly different.

SUMMARY.

The swelling of isoelectric gelatin added to various volumes of acid of different concentration at $5^{\circ}C.$ has been determined. The swelling is determined only by the concentration of the supernatant solution, at equilibrium and is independent of the volume of acid.

Similar experiments with *unpurified* gelatin show that in this case, owing to the presence of neutral salts the swelling is a function of the volume as well as the concentration of acid.

Both results are predicted by the Procter-Wilson-Loeb theory of the swelling of gelatin.

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A METHOD FOR THE DETERMINATION OF DIFFUSION CONSTANTS AND THE CALCULATION OF THE RADIUS AND WEIGHT OF THE HEMOGLOBIN MOLECULE.

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The diffusion coefficient of a substance in solution is a useful constant since from it may be calculated, by Einstein's equation (1), the radius and weight of the particle. It is particularly useful in the case of substances which cannot be obtained in pure solution and which are frequently of interest in biological work. In such cases the diffusion coefficient is the only practical means of determining the molecular size or weight. The classical method for determining the diffusion coefficient is so beset with experimental difficulties and, in the case of slow moving substances, requires such a long time that it is useless for most biological material.

It occurred to the writers that if the diffusion could be made to occur across a thin porous plate the process would be greatly accelerated owing to the high concentration gradient, and at the same time the elaborate precautions to prevent convection currents, etc. inherent to the classical method would be eliminated (2). Search of the literature revealed that several attempts (3-6) had already been made in this direction, but none were practical for biological material owing to the unsuitable nature of the membranes used. It was found, after a number of trials, that rapid and reproducible results could be obtained with thin membranes made either of alundum or glass powder.

Theory of the Method.—The diffusion coefficient is defined as the quantity of material that will diffuse across a plane of unit dimensions in unit time under unit concentration gradient, or

$$(1) \quad D = \frac{dQ}{A \, dt \, \frac{dc}{dx}}$$

where D is the diffusion coefficient, dQ is the quantity which passes across the plane of area A in time dt under a concentration gradient of dc/dx . There are a number of solutions of this differential equation depending on the conditions of the experiment (*cf.* Mellor (7)). The present case is in many respects the simplest. Suppose a solution of concentration C_1 is separated from a more dilute solution of concentration C_2 by a porous membrane through the pores of which the solute can diffuse. Both solutions are stirred so that diffusion occurs only across the membrane. The solute will diffuse from C_1 to C_2 and if the volume of these solutions is relatively large and the experiment carried on for a short time the concentrations will remain practically constant.* Let the effective area of the pores be A and the effective

* The equation for diffusion, under the above conditions, for the general case in which the concentration cannot be considered constant during the duration of the experiment, may be derived as follows:

Let

S = total solute at beginning of experiment

v_1 = volume concentrated solution

v_2 = volume dilute solution

Q = quantity solute in dilute solution

$$= c_2 v_2$$

$$S - Q = c_1 v_1$$

From equation (1)

$$\frac{dQ}{dt} = \frac{DA}{h} (c_1 - c_2)$$

substituting for c_1 and c_2

$$= \frac{DA}{h} \left(\frac{S - Q}{v_1} - \frac{Q}{v_2} \right)$$

or

$$= \frac{DA}{h v_1 v_2} (v_2 S - (v_2 + v_1) Q)$$

Integrating, substituting $K = \frac{h}{A}$

$$D = \frac{2.3 K v_1 v_2}{(v_1 + v_2) t} \log \frac{v_2 S - (v_2 + v_1) Q_0}{v_2 S - (v_2 + v_1) Q}$$

where $Q = Q_0$ when $t = 0$.

length (the distance through which the solute diffuses) be h . The concentration gradient will then be constant and equal to $\frac{C_1 - C_2}{h}$ and the quantity Q diffusing in time t will be simply

$$(2) \quad Q = D A t \frac{C_1 - C_2}{h}$$

or

$$(3) \quad D = \frac{h Q}{A t (C_1 - C_2)}$$

If the experiment is so arranged that the dilute solution is originally pure solvent, C_2 is zero and the equation is still further simplified to

$$(4) \quad D = \frac{h Q}{A t C_1}$$

Dimensions of D .—Since concentration may be expressed as quantity per unit of volume, the units used to measure the quantity cancel out provided the same unit is used to express the concentration as is used to measure the quantity diffusing. D therefore reduces to area over time, or if time is expressed in days and length and volume in centimeters, to cm.^2 per day. This may be seen from the following equation

$$(5) \quad D = \frac{h \text{ cm.}}{A \text{ cm.}^2} \frac{Q \text{ units}}{t \text{ day } \frac{Q_1 \text{ units}}{\text{cm.}^3}} = \frac{h Q \text{ cm.}^3}{A Q_1 t \text{ day}}$$

in which Q_1 is the number of units per cc. of the concentrated solution. If the amount contained in 1 cc. of the concentrated solution is taken as the unit of quantity, *i.e.* if $Q_1 = 1$, and the amount diffused is expressed in this unit (*i.e.* as the number of cc. of the concentrated solution containing the quantity diffused), the equation may be still further simplified and written

$$(6) \quad D = \frac{h Q_{\text{cc.}} \text{ cm.}^3}{A t \text{ day}} = \frac{K Q_{\text{cc.}} \text{ cm.}^3}{t \text{ day}}$$

where $Q_{\text{cc.}}$ is the number of cc. of the concentrated solution that contains the amount of substance diffused. For instance, if it were found in an

experiment with HCl that 10 cc. of 0.10 acid had diffused and the concentrated solution were 0.10 M acid then $Q_{cc.}$ would be 10. If the concentrated solution had been 1.0 M, $Q_{cc.}$ would be 1.0, etc. Any comparative unit of quantity may therefore be used to express the quantity diffused provided the concentration is expressed in the same units and this is an advantage in dealing with biological material.

It is evident that in order to obtain the diffusion coefficient in absolute units it is necessary to know the dimensions of the membrane through which the diffusion occurs. In the case of porous membranes this value cannot be measured directly since the effective radius and the arrangement of the pores is not known. For any one membrane, however, the effective thickness and area may be assumed constant and therefore h/A is constant and may be called K , the membrane constant. In order to obtain this value it is necessary to standardize the apparatus against some solution the diffusion constant of which is known, just as is the case with a conductivity cell. K , the cell constant, may then be found from the equation

$$(7) \quad K = \frac{D \, t}{Q_{cc.}}$$

When this constant has been determined for a particular membrane it may then be used to determine the diffusion coefficient for unknown substances, provided of course that the effective pore area is the same for the standard and for the unknown.

Construction of the Apparatus.—It follows from the preceding considerations that the following conditions must be fulfilled by the apparatus.

1. The concentration of the two solutions on opposite sides of the membrane must be kept constant, by stirring or otherwise, so that diffusion occurs only across the membrane.

2. The quantity allowed to diffuse must be small enough so that the difference in concentration between the two sides may be considered constant during the experiment.

3. The membrane must be thick enough so that the liquid in the pores is not disturbed by the stirring of the solutions. On the other hand, the thinner it is the more rapidly the experiment can be completed.

4. The pores must be small enough to prevent convection currents in the liquid held in them and large enough to allow free diffusion of the particles (or molecules) of the solute.

5. The membrane must be level in order to prevent flow from one solution to the other.

It was found after a number of trials that porous glass or alundum discs gave satisfactory results.

The Membranes.—Filter discs made by pressing together a uniform powder of Jena glass are obtainable from the Jenaer Glaswerk, Schott and Gen., Jena, Germany—American agent J. E. Bieber, 1123 Broadway, New York. Discs of the porosity now called No. 4, but formerly designated as <7, were used. According to the manufacturers the pore diameter is 5 to 10 microns or thousandths of a millimeter. The radius of the hemoglobin molecule is about 2.7×10^{-3} microns. The discs are cut down to approximately the desired diameter with an ordinary hack saw and rounded off exactly on a carborundum wheel. Then the pores of the membrane are filled with melted rosin by gentle suction applied through the stop-cock of the glass vessel to which the disc is to be attached. This is to prevent the clogging of the pores with the carborundum powder with which the disc is next ground down to about 0.5 mm. thickness. At the beginning the grinding is done on a carborundum stone, and finished on a flat piece of glass. The rosin is removed with a suitable solvent such as chloroform.

The glass discs are usually sold already fused on to vessels of various shapes, one of them very like the one used in these experiments. It is better, however, to start with the loose discs, since as a result of the fusion the effective area of the disc is lessened.

The membranes are attached to the glass vessel with de Khotinsky cement applied to the bottom of the vessel. Then the edges of the discs which may stick out slightly here and there are carefully ground down even with the vessel and covered with cement. Leaks are tested for by blowing through the stop-cock while the membrane is under water.

Alundum filter discs, porosity RA 225, are made by the Norton Company, Worcester, Massachusetts. The disc is ground down to the desired diameter on a wheel and to the desired thickness by being rubbed against another disc. At the finish a fresh flat disc should be used. After the powder produced in the grinding is washed out the disc is attached.

Some batches of alundum discs contain air spaces large enough to cause small holes when the discs are ground thin. Such discs cannot be used.

Assembly of the Apparatus.—The apparatus is assembled as shown in Fig. 1. The cell contains about 20 cc. and the outside solution is 10 to 50 cc. depending on the rate of diffusion, the time of the experiment, and the analytical method used. The cell is filled by gentle suction through the membrane and rinsed with water. It is then clamped in position and leveled by means of a small level or by placing a dish of mercury just below it. The membrane is made parallel to the mercury by adjusting the universal clamp. The mercury is then removed and replaced with sufficient pure solvent to cover the membrane. The time is noted and the diffusion allowed to continue until the desired amount has passed through

the membrane. The outside solution is then pipetted off and analyzed and replaced by a new portion. This process is continued until the quantity passing through the membrane is constant for equal time intervals, *i.e.*, the system is in the steady state. This constant figure is then used. If more than a few per cent of the solute diffuses out it is necessary to correct the concentration of the inside solution by this amount. The amount diffusing during any one determination should be not more than 2 or 3 per cent of the total in the concentrated solution, and under these conditions a sufficiently accurate correction is obtained by con-

sidering the concentration as the average value for the duration of the experiment. For instance, if it is found that 4 per cent of the solute has dialyzed out during the experiment, then the concentration of the inside solution has dropped from 100 to 96 during the experiment. For purposes of calculation it is considered to have remained constant at 98 per cent.

The arrangement shown in which the heavier solution is above obviates the necessity of stirring since, as the solute diffuses through, a solution is formed near the membrane which is heavier than the pure solvent and so sinks to the bottom. This process could be clearly observed in the experiments with hemoglobin. As the solution in contact with the upper side of the membrane becomes more dilute it is replaced by the heavier concentrated solution above it. There are also slight convection currents due to temperature effects and vibrations from the stirrer of the water bath which aid this process. The fact that even with HCl the quantity diffusing remains constant for constant time intervals shows that the two solutions must remain nearly homogeneous,

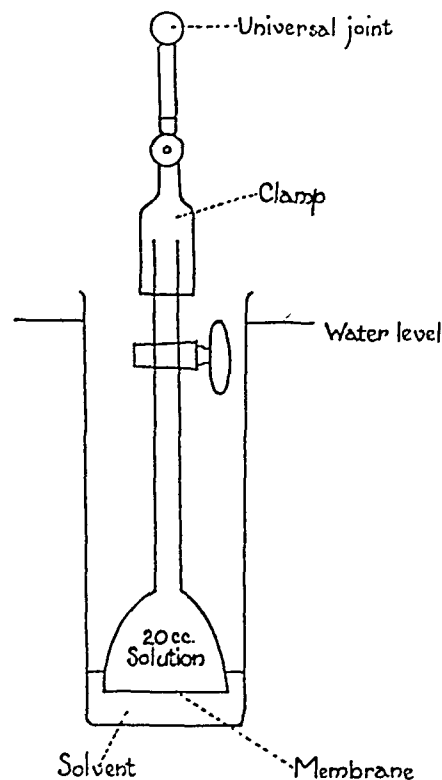


FIG. 1. Apparatus for determination of diffusion coefficients.

i.e., that diffusion occurs only across the membrane as is assumed in the calculation. Any flow across the membrane would result in variable results in different experiments and would cause an increase in the relative amount of solution passing the membrane with more concentrated solutions.

Evidently the change in concentration due to the diffusion would be less if the volumes were larger. However, a larger volume of the cell increases the error caused by temperature fluctuations and a large volume of outside solution renders

the analysis more difficult. The apparatus is placed in a water bath at 5°C. since the coefficient of expansion of water is near the minimum at this temperature and also since biological material is more stable at this temperature. The temperature regulation was obtained by placing an ordinary electrically regulated glass water bath in a refrigerating room which had a temperature of about 3° to 4°C.

Calibration of the Apparatus.—It was stated above that since the actual dimensions of the membrane are not known it is necessary to standardize the apparatus by means of a solution whose diffusion coefficient is known. HCl, lactose, and several salts were used for this purpose and it was found that the same cell constant was obtained for any particular membrane with all substances. The results with HCl will be given as an example.

The results of the experiment are shown in Table I. It was found that the constant for the cell was 0.148. The physical significance of this figure, as may be seen from equation (6), is evidently the ratio of effective thickness to pore area of this membrane.

TABLE I.
Diffusion of HCl through Alundum Membrane 1, 5°C.

Solution	Time <i>t</i>	Equivalent cc. 0.1 N HCl diffused	Average
0.1 N HCl	<i>days</i>	<i>Q_{cc.}</i>	0.261
	0.0208	0.262	
	.0208	.258	
	.0208	.262	

$$K = \frac{Dt}{Q_{cc.}} = \frac{1.85 \times 0.0208}{0.261} = 0.148$$

D = diffusion coefficient 0.1 N HCl at 5°, extrapolated from Ohölm's data = 1.85 cm.² per day.

It is now possible to determine the diffusion coefficient of another substance by means of this membrane. Carbon monoxide hemoglobin was chosen for this purpose since the molecular weight of hemoglobin is known from osmotic pressure measurements and the weight calculated from the diffusion experiment may therefore be checked against this value.

Preparation of Hemoglobin.—Whipped beef blood is saturated with CO, cooled, and washed three times with cold isotonic salt solution which is likewise saturated with CO. An equal volume of water is added to the corpuscles. The laked corpuscles are shaken with a quarter their volume of toluene (Heidelberger (10)) and the mixture allowed to stand in the cold overnight. The toluene and cell debris are removed by centrifuging. By dilution with 1/20 M phosphate or borax buffers the hemoglobin is brought to the desired concentration and hydrogen ion concentration. It is filtered through fine filter paper and saturated with CO.

During the experiments the hemoglobin is allowed to diffuse into buffer solution saturated with CO.

The carbon monoxide hemoglobin which has diffused through the membrane is estimated colorimetrically.

Carbon monoxide hemoglobin was used since it is the most stable form of hemoglobin.

TABLE II.

Determination of Diffusion Coefficient of Hemoglobin, 5°C.

Membrane	Hemoglobin solution	Time	Hemo- globin diffused as cc. con- centrated solution	K for membrane	D hemoglobin cm. ² /day
		days	Q _{cc.}		
Alundum 1	1 per cent pH 6.8	0.75	0.202	0.150	0.0405
		.92	.264		.0430
		2.06	.558		.0407
	2.5 per cent pH 6.8				.0430
					.0412
					.0428
Alundum 2	2.5 per cent COHb pH 6.8			.168	.0401
	2.5 per cent COHb M/20 borax pH 9.3				.0397
Glass 1	2.5 per cent COHb pH 6.8			.100	.0434
Glass 2	2.5 per cent COHb pH 6.8 M/20 phosphate			.0833	.0456
Average.....					0.0420 ± 0.0005

Diffusion Coefficient of Hemoglobin.—The results of several diffusion experiments are given in Table II. The table gives the amount of hemoglobin found to have diffused through various membranes after various time intervals and from different hemoglobin solutions. This quantity has been expressed as the number of cc. of the concentrated solution (used for the experiment), which contained the same amount

of hemoglobin. The diffusion coefficient of hemoglobin may therefore be calculated from these figures by means of equation (8).

$$(8) \quad D = \frac{K Q_{cc.}}{t}$$

The values obtained in this way are given in the last column of the table. The average value is 0.0420 ± 0.0005 cm.² per day.

Calculation of the Radius and Molecular Weight of the Molecule from the Diffusion Coefficient.—According to Einstein the diffusion coefficient is related to the radius of the molecule by the following equation

$$(9) \quad D = \frac{R T}{N} \cdot \frac{1}{6 \pi r \eta}$$

$$R = 8.3 \times 10^7 \text{ erg} \cdot \text{deg.}^{-1} \cdot \text{mole}^{-1}$$

$$N = 6.06 \times 10^{23} \text{ mole}^{-1}$$

$$T = 278 \text{ deg.} - \text{for } 5^\circ\text{C.}$$

$$r = \text{radius of particle including water of hydration, in cm.}$$

$$\eta = \text{viscosity of water at } 5^\circ\text{C.}$$

$$= 0.01519 \text{ gm. cm.}^{-1} \text{ sec.}^{-1}$$

$$= 0.01519 \text{ erg} \cdot \text{sec. cm.}^{-2}$$

$$D = \frac{1.33 \times 10^{-12}}{r} \frac{\text{erg} \cdot \text{deg.} \cdot \text{mole} \cdot \text{cm.}^2}{\text{erg} \cdot \text{deg.} \cdot \text{mole} \cdot \text{cm.} \cdot \text{sec.}}$$

$$= \frac{1.33 \times 10^{-12} \text{ cm.}^2}{r \text{ sec.}}$$

or for H₂O at 5°C.

$$(10) \quad = \frac{1.148 \times 10^{-8} \text{ cm.}^2}{r \text{ day}}$$

therefore

$$(11) \quad r = \frac{1.148 \times 10^{-8}}{D}$$

$$= 2.73 \times 10^{-7} \text{ cm.}$$

The molecular weight of the particle may be calculated from the radius by the following equation provided the specific gravity is known.

$$\begin{aligned}
 (12) \quad m &= \frac{4}{3} \pi r^3 g N \\
 g &= \text{specific gravity} = \text{gm. cm.}^{-3} \\
 &= 1.33 \\
 &= \frac{4}{3} \times 3.14 \times 6.06 \times 10^{23} g r^3 \\
 &= 25.4 \times 10^{23} \cdot g \cdot r^3 \\
 &= 25.4 \times 10^{23} \times 1.33 \times 20.2 \times 10^{-21} \\
 &= 68,500 \pm 1,000
 \end{aligned}$$

The specific gravity is assumed to be the same as that of solid hemoglobin and the calculation gives a molecular weight of $68,500 \pm 1,000$ which agrees within the experimental error with that of 67,000 found by Adair (8) by osmotic pressure measurements, and of 68,000 found by Svedberg (11) from experiments on the rate of sedimentation.

Einstein's equation assumes that the solute molecules are large compared to the solvent molecules and therefore could not be used for substances of small molecular weight. It was found by Euler (9), however, that for such molecules the diffusion coefficient is inversely proportional to the square root of the molecular weight, *i.e.*

$$(13) \quad \frac{D_1}{D_2} = \sqrt{\frac{m_2}{m_1}}$$

In such cases the molecular weight could be obtained by simple proportion from the diffusion constant of some known substance of about the same molecular weight.

Sources of Error in the Method and Assumptions Underlying the Theory.—The effect of temperature, leveling, and change of concentration during the experiment have already been mentioned. It is conceivable that the substance might stick to the membrane and so fill up the pores, or that some of the pores would not allow the unknown solution to pass while the solution used as a standard could pass. For this reason it is advisable to use different types of membrane since it is

unlikely that the effect would be the same in membranes made of different material. Another method of testing for this error is to determine the effect of filtering the solution through the membrane. Any appreciable adsorption would be shown by a change in concentration on filtering.

Einstein's equation for calculating the radius and molecular weight from the diffusion coefficient is based on the following assumptions. The diffusing particles (molecules) are few and large compared to those of the solvent. They are spherical and are impelled by a force equal to the osmotic pressure as given by Van't Hoff's law against a resistance as given by Stokes' law. The work of Perrin and others has shown that Einstein's equation is valid for a number of colloidal solutions. The specific gravity of the particle, however, is more uncertain since it might very well consist partially of water and so be lighter than the dry solid in bulk.

In the case of ionized substances another complication is introduced since the diffusion rate of one ion is influenced by the accompanying ion. Most biological substances, however, are weak electrolytes so that the quantity present in the form of ions is usually small. It is conceivable in the case of such things as enzymes that several different size molecules might be determined simultaneously by the method of analysis used. In this case no constant diffusion coefficient would be obtained since the smaller molecules would diffuse first. If the proportion or the relative number and sizes of the different molecules were known this effect could be corrected for, at least in theory. Practically, however, decreasing coefficients found on succeeding diffusions would indicate a mixture of substances but could hardly be analyzed further in a quantitative way. The problem is evidently the same as the separation of gases by diffusion.

Finally, it may be said that the molecular weight obtained in this way is that defined by the properties of the solution and used by the physicist and physical chemist.

SUMMARY.

A method is described for determining the diffusion coefficient of solutes by determining the rate of passage of the solute through a thin porous membrane between two solutions of different concentration.

The method has been used to determine the diffusion coefficient of carbon monoxide hemoglobin. This was found to be 0.0420 ± 0.0005 cm.² per day at 5°C.

The molecular weight of carbon monoxide hemoglobin calculated by means of Einstein's equation from this quantity is $68,600 \pm 1,000$.

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OSMOSIS OF LIQUIDS. II.

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INTRODUCTION.

We take an osmotic system

$$n_1 \times L_1 | n_1' \times L_1' \quad (1)$$

in which on the left side of the membrane are n_1 quantities of a liquid L_1 and on the right side n_1' quantities of L_1' . We now assume, in the same way as in the preceding communication,¹ that the system contains three substances, *viz.* X , Y and W (W = water); then we may represent L_1 and L_1' by the points 1 and 1' (Fig. 3, Paper I). The position of their complex e , as we have seen previously, is determined by:

$$e1 : e1' = n_1' : n_1 \quad (2)$$

When the membrane transmits only W , during the osmosis L_1 will proceed along the line 1.4 (Fig. 3, Paper I) and L_1' along the line 1'.4'. At last the osmotic equilibrium forms:

$$n_1 \times L_1 | n_1' \times L_1' \quad (3)$$

Then the water diffusion has stopped; the two liquids are situated on the same isotonic W -curve ab and consequently they have the same O.W.A.

The phenomena are different, however, when the membrane transmits two or three substances. Here we shall discuss only the membrane that transmits the three substances.

¹ Schreinemakers, F. A. H., *J. Gen. Physiol.*, 1928, xi, 701.

I.

The Theoretical Osmosis Path.

We represent the liquids of system (1) by the points 1 and 1' in Fig. 1; in order to save place, the sides and angle points of the triangle *WXY* have been omitted. The complex of both the liquids is represented by point *e*; its position has been determined by (2).

As soon as the osmosis has begun and the substances *X*, *Y* and *W* diffuse through the membrane in some direction or other, the liquids change their compositions. After a time t_2 the left side liquid has arrived e.g. in point 2 and the right side liquid in point 2' (Fig. 1). As the quantities of the liquids have also changed now, we represent them by n_2 and n_2' . Consequently system (1) has now passed into:

$$n_2 \times L_2 \mid n_2' \times L_2' \quad (4)$$

Of course the total quantity of the two liquids remains constant during the osmosis; we have, therefore: $n_2 + n_2' = n_1 + n_1'$. The total composition of the system also remains constant; consequently the line 2.2' also runs through point *e*. Instead of (2) we now have however:

$$e2 : e2' = n_2' : n_2 \quad (5)$$

After a time t_3 the left side liquid has arrived in a point 3 and the right side liquid in a point 3'; we then have a system:

$$n_3 \times L_3 \mid n_3' \times L_3' \quad (6)$$

For this we obtain:

$$n_3 + n_3' = n_2 + n_2' = n_1 + n_1'$$

The line 3.3' also runs through point *e*; we now have:

$$e3 : e3' = n_3' : n_3 \quad (7)$$

Of course the same obtains for all systems that have formed during the osmosis. At the end of the osmosis the two liquids must be in osmotic equilibrium with one another. If only one substance passes through the membrane, then, as we have seen in Paper I, the two final liquids are different. If, however, the membrane is permeable with

respect to all substances, then the same final liquid must form on both sides of the membrane;² it is clear that this liquid is represented by point e . At the end of the osmosis, therefore, we get the system:

$$L_e | L_e \quad (8)$$

During the osmosis the left side liquid, therefore, proceeds along a curve $1.2.3 \dots e$, the right side liquid along a curve $1'.2'.3' \dots e$; the arrows indicate the direction in which the liquids proceed along these curves. We call these curves "the osmosis path" of the left and right side liquids; together they form the "osmosis path" of the system. In Figs. 2 and 4 osmosis paths have been drawn, which have

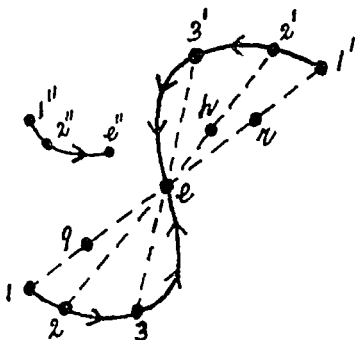


FIG. 1

been determined experimentally and will be more fully discussed later on. We shall call liquids, present at the same moment on both sides of the membrane (e.g. 1 and 1' or 2 and 2' etc.), "conjugated" liquids; the points representing those liquids are called conjugated points; the lines, uniting two conjugated points (e.g. 1.1' or 2.2' etc.) are called conjugation lines. Therefore, the conjugation lines of an osmosis path all run through a point e , which represents the composition of the complex and consequently of the final liquid too. Reciprocally every

² We here assume that no unmixing into 2 or more liquids will occur in the system.

straight line, going through point e , is a conjugation line; the points of intersection represent two liquids, to be found at a certain moment on both sides of the membrane.

The osmosis path of system (1) starts in Fig. 1 from the points 1 and 1'; its form, however, depends on several other factors, some of which we are going to discuss.

(a) The form of the path depends on $n_1:n_1'$ viz. on the ratio of the quantities of the liquids, which are brought on both sides of the membrane. For if we alter this ratio, it will be seen from (2) that the position of final point e on line 1.1' also changes; we imagine this e.g. in q (or r). Of course the new osmosis path which must now run through q (or r) has another form than the path running through point e . Consequently an infinite number of paths start from the points 1 and 1' determined by the ratio $n_1:n_1'$; we are able to prove that these paths touch one another in the points 1 and 1'.

(b) The form of the path depends on the direction in which and the velocity with which the substances diffuse through the membrane at any moment; consequently this form also depends on the nature of the membrane used and will, therefore, differ with different membranes. Later on we shall make this clear with some examples and discuss it in connection with other phenomena.

II.

The Experimental Osmosis Path.

If we leave an osmotic system

$$n_1 \times L_1 \mid n_1' \times L_1' \quad (9)$$

in which the two liquids and their quantities have been absolutely determined, alone, then it will change in a definite way. At a moment t_2 we then have a system

$$n_2 \times L_2 \mid n_2' \times L_2' \quad (10)$$

in which again the two liquids and their quantities have been completely determined. Consequently the system proceeds along a definite path (1.2.3. e and 1'.2'.3'. e of Fig. 1); we call this the "theoretical" path. If, however, in order to find out the composition of the

liquids of (10) we take away a little of these liquids, then we have instead of (10) the new system:

$$(n_1 - q) \times L_1 | (n_2 - q') \times L_2' \quad (10a)$$

q and q' represent the quantities, taken away. Although (10) and (10a) consist of the same liquids, they still have another final liquid as a rule. Of course that of (10) is situated in point e ; that of (10a) is situated in a point h on the conjugation line 2.2' (Fig. 1).

In accordance with (5) for e we obtain:

$$e2 : e2' = n_2' : n_2$$

For point h we obtain, however:

$$h2 : h2' = (n_2' - q') : (n_2 - q)$$

Consequently e and h are different points, unless accidentally:

$$q : q' = n_2 : n_2'$$

System (10) which we no longer have, would, therefore, proceed along the paths 2.3. e and 2'.3'. e ; system (10a) which we do have proceeds, however, along the paths 2. h and 2'. h which have not been drawn; consequently both systems proceed along different paths, which touch one another, however, in the points 2 and 2'. (Cf. paragraph (a).) Of course the conjugation lines of system (10) all run through point e , those of (10a) through point h .

If we take in (10) *e.g.*, $n_2 = 240$ and $n_2' = 260$, then we have the system:

$$240 \times L_1 | 260 \times L_2'$$

If we take away 5 gm. on both sides, then we get:

$$235 \times L_1 | 255 \times L_2'$$

For the points e and h we obtain, therefore:

$$e2 : e2' = 260 : 240 = 1.083 : 1$$

$$h2 : h2' = 255 : 235 = 1.085 : 1$$

In Fig. 1 point h must be situated very closely to e ; consequently the paths of both systems will practically coincide. As we have to

take away a little of the liquids now and then for the experimental determination of a path, we consequently do not define the theoretical path, but a succession of parts of theoretical paths; they pass into one another continually in the points which have been determined experimentally. We call this path the "experimental." The less we change the ratio of the two liquids in taking away little quantities of them, the less the experimental will differ from the theoretical path.

III.

The Path of the Membrane.

As the substances X , Y and W diffuse, the membrane must also contain these three substances; consequently we may say that the membrane contains a liquid, the composition of which changes continually during the osmosis. If in Fig. 1 we represent this liquid by a point, then, during the osmosis, this proceeds along a curve ($1''.2''.e''$ Fig. 1); therefore, we call this the "path of the membrane."

Consequently we have to distinguish three paths, *viz.* the path of the left side liquid ($1.2.e$), the path of the right side liquid ($1'.2'.e$) and the path of the membrane ($1''.2''.e''$). Of course the final liquid e'' of the membrane generally has another composition than the final liquid e . Therefore, the theoretical and experimental paths of a system depend besides upon the quantity and the composition of the liquid absorbed by the membrane. If, however, we have a small and thin membrane and much liquid, this influence may be neglected; if, however, we have a large or thick membrane, this influence may be very strong. We shall refer to this later on.

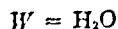
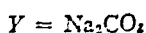
IV.

Some Examples.

Now we shall discuss some paths which we have determined in the systems:



The right side liquid consists of:



On the left side of the membrane is pure water, which, however, takes in X and Y at once and passes into a ternary liquid. The membrane of these systems consisted of a pig's bladder, which had been degreased with ether. The results of the determinations³ are found in the Tables I-IV. In the first column of I-III we find the number of the determination under t , the time, *vis.* the number of hours after

TABLE I.

	t	X	Y	X	Y
1	0	0	0	6.991	9.021
2	7	1.263	1.423	5.802	7.732
3	19	2.242	2.388	4.992	6.950
4	31	2.739	3.109	4.536	6.452
5	51	3.316	3.533	4.195	6.123
6	91	3.646	4.216	3.877	5.665
7	118	3.682	4.410	3.803	5.437

TABLE II.

	t	X	Y	X	Y
1	0	0	0	6.729	9.057
2	17	1.246	0.387	5.663	8.268
3	29	2.756	1.163	4.557	7.430
4	41	3.589	1.930	3.876	6.856
5	69	$\frac{3.823}{q}$	2.763	$\frac{3.762}{q'}$	6.400
6	101	$\frac{4.179}{q}$	3.547	3.792	5.988
7	143	4.138	4.319	3.843	5.688
8	311	3.934	5.260	3.901	5.378

the beginning of the osmosis. We find in the third and fourth columns under X the amount of NaCl and under Y the amount of Na_2CO_3 of the left side liquid; in the fifth and sixth columns we find the same for the right side liquid. The concentrations have been given in per cent of weight. As the W amount of the liquids follows at once from the X and Y amounts, this has not been given.

³ They have been done in collaboration with G. M. A. Kayser and L. J. van der Wolk.

System I (Table I).—If with the aid of Table I we draw the osmosis path of this system, we get a path such as has been represented schematically by $W2e$ and $1'.2'.e$ in Fig. 2. The left side path We is situated below, the right side path $1'e$ above the conjugation line $W1'$. We see from Table I and Fig. 2 that the X and Y amount of

TABLE III.

	t	X	Y	X	Y
1	0	0	0	9.222	8.976
2	4	0.377	0.087	8.781	8.811
3	12	1.223	0.334	8.024	8.489
4	24	2.366	0.695	7.149	8.056
5	36	3.319	1.054	6.494	7.685
6	54	4.391	1.640	5.852	7.211
7	78	5.212	2.417	5.431	6.702
		$\text{---}q$		$\text{---}q'$	
8	108	†5.666	3.258	5.262	6.206
9	145	5.651	4.051	†5.260	5.903
10	193	5.551	4.660	5.278	5.650
11	240	5.478	5.024	5.308	5.534

TABLE IV.

	X	Y	Membrane	Type
1 (I)	6.991	9.021	H^a	I
2	7.363	9.194	H^b	I
3	9.238	9.165	H^c	I
4 (II)	6.729	9.057	VI^o	II
5	12.006	7.019	VI^i	II
6 (III)	9.222	8.976	VI^h	III
7	9.227	8.984	VI^d	II or III
8	4.681	10.107	VI^e	II or III

the left side liquid continually increases during the osmosis; that of the right side liquid decreases. Calculating the W amount, we find that the W amount of the left side liquid continually decreases during the osmosis; that of the right side liquid increases.

We may represent the progress of the osmosis still in another way. For this purpose we plot the time t of the osmosis on a horizontal axis

(Fig. 3) with the aid of Table I; on the vertical axis we may plot either the X , the Y or the W amount of the two liquids. If we plot the X amount, we get an $X.t$ diagram such as has been drawn schematically in Fig. 3. The fully drawn curve $0.2.3.e$ obtains for the left side liquid, the dotted curve $1'.2'.3'.e$ for the right side liquid. The final point e of the two paths is situated at infinite distance; consequently both paths approach one another asymptotically. With the aid of Table I the reader can easily draw the $Y.t$ and $W.t$ diagram of this system himself. We can also summarize the previous considerations in a scheme.

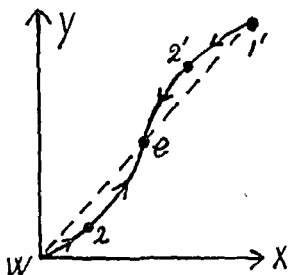


FIG. 2

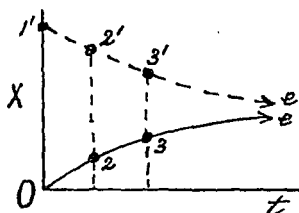


FIG. 3

Scheme I.

X	Y	W	
$\uparrow < \downarrow$	$\uparrow < \downarrow$	$\downarrow > \uparrow$	(12)

This means (*cf.* Paper I) that the left side liquid has a smaller X and Y amount, but a larger W amount than the right side liquid. An arrow pointing upwards indicates that the concentration increases; an arrow pointing downwards indicates that the concentration decreases. This shows therefore, that during the osmosis the X and Y amounts of the left side liquid increases, that of the right side liquid decreases; the W amount of the left side liquid decreases, that of the right side liquid increases. Consequently during the osmosis the liquids change (Paper I) their X , Y and W amounts normally.

System II (Table II).—If we draw the osmosis path of this system with the aid of Table II, we get the curves $W.q.5.6.e$ and $1'.q'.5'.6'.e$ of Fig. 4; the $X.t$ diagram has been represented in Fig. 5. For the sake of clearness both figures have been strongly schematised.

It appears from Table II that during the osmosis the X amount (consequently NaCl) of the left side liquid at first increases till the determination marked \dagger (*viz.* 4.179 per cent NaCl) and decreases afterwards. Consequently in the vicinity of $n^\circ = 6$ the left side liquid gets a maximum X amount; we shall plainly say that this maximum coincides with point 6. Consequently the left path $Wq56e$ must have

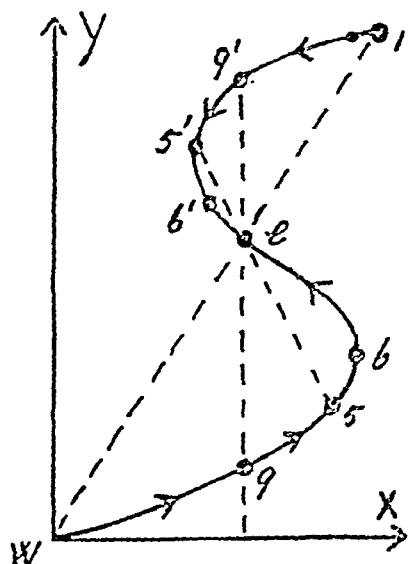


FIG. 4

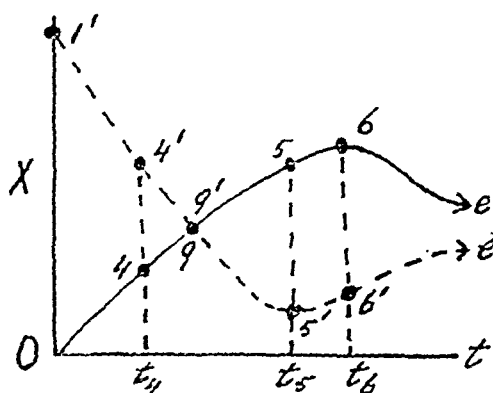


FIG. 5

a vertical tangent in point 6 of Fig. 4; in point 6 of Fig 5 this tangent is horizontal. The X amount of the right side liquid at first decreases till the determination marked \dagger (*viz.* 3.762 per cent NaCl) and afterwards increases. Therefore, in the vicinity of $n^\circ = 5'$ the right side liquid gets a minimum X amount; again we assume that it coincides with point 5'. The right path $1' q' 5' 6' e$ must, therefore, have a vertical tangent in point 5' of Fig. 4 and a horizontal tangent in point 5' of Fig. 5. It also appears from Table II that between the determinations 4 and 5 the left side and the right side liquids have had the same X amount. This has been indicated in the table by the hori-

zontal lines beside which the letters q and q' have been placed. Consequently in Fig. 4 the conjugation line qeq' is vertical; of course in Fig. 5 q and q' are situated in the point of intersection of the two curves.

It appears from Table II or Fig. 4 that the changes of the Y and W amounts (*viz.* Na_2CO_3 and H_2O) during the total osmosis may be represented by

$$\begin{array}{cc} Y & W \\ \uparrow < \downarrow & \downarrow > \uparrow \end{array} \quad (13)$$

just as in system I the liquids, change their Y and W amounts normally.

The X amount, however, changes in an absolutely different way from that in system I; we see this at once from the $X.t$ diagrams (Figs. 3 and 5) and from the osmosis paths (Figs. 2 and 4). As long as the system remains in the part Wq (and $1'q'$) of its path, the X amount on the left side of the membrane is smaller than on the right side; as during the osmosis it increases on the left side and decreases on the right side, we consequently have the symbol:

$$Wq (1'q') \quad \uparrow < \downarrow \quad (\text{Figs. 4, 5}) \quad (14)$$

So in this part of the path the X amount changes as in system I *viz.* normally-normally.

If, however, the left side liquid comes in q and the other consequently in q' , both get the same X amount. As this increases on the left side of the membrane and decreases on the right side, we represent it by:

$$q (q') \quad \bullet \uparrow \equiv \downarrow \bullet \quad (\text{Figs. 4, 5}) \quad (15)$$

As we may consider the change on both sides of the membrane as abnormal, an asterisk has been placed with both arrows.

So here we have the same X amount on both sides of the membrane; during further osmosis this changes again, becoming equal once more in point e at the end of the osmosis.

When the system is in part $q5$ (and $q'5'$) of its path, then the X amount on the left side is greater than that on the right side. As,

however, it is still increasing on the left side and decreasing on the right side, we have the symbol:

$$q5 \ (q'5') \quad * \uparrow > \downarrow * \quad (\text{Figs. 4, 5}) \quad (16)$$

Consequently the X amount changes abnormally here on both sides.

When the system is in point 5 (and 5') then the X amount on the right side of the membrane (*viz.* in point 5') is a minimum; therefore, this X amount does not change in an infinitely small time dt ; on the

SCHEME II.

		X	Y	W
Wq	(Wq')	$\uparrow < \downarrow$	$\uparrow < \downarrow$	$\downarrow > \uparrow$
q	(q')	$* \uparrow = \downarrow *$	"	"
$q5$	$(q'5')$	$* \uparrow > \downarrow *$	"	"
5	$(5')$	$* \uparrow > \downarrow *$	"	"
5.6	$(5'.6')$	$* \uparrow > \uparrow$	"	"
6	$(6')$	$* \downarrow > \uparrow$	"	"
6.e	$(6'.e)$	$\downarrow > \uparrow$	"	"

left side of the membrane it increases, however. We represent this by:

$$5 \ (5') \quad * \uparrow > | * \quad (\text{Figs. 4, 5}) \quad (17)$$

in which the dash indicates that the right side X amount does not change in a time dt .

When the system is in part 5.6 (and 5'.6') of its path, the X amount increases on both sides. Consequently we have the symbol:

$$5.6 \ (5'.6') \quad * \uparrow > \uparrow \quad (\text{Figs. 4, 5}) \quad (18)$$

Consequently, the X amount changes abnormally here on the left side and normally on the right side.

If we also consider the system in point 6 (and 6') and in part 6.e (and 6'.e) of its path and combine all symbols, we get scheme II. From this we learn among other things the following: At the beginning of the osmosis the X amount of the left side liquid is smaller, afterwards (in q and q') equal, next larger and at the end of the osmosis (point e) again equal to that of the right side liquid. The X amount of the

left side liquid increases, beginning at point W as far as 6, and decreases afterwards as far as e . The X amount of the right side liquid decreases, starting from point $1'$ as far as $5'$, and increases afterwards as far as e . The X amount of the left side liquid changes abnormally from q to 6, for it increases, although it is already greater than that of the other liquid. The X amount of the right side liquid changes abnormally from q' to $5'$, for it decreases although it is already smaller than that of the other liquid. On part 5.6 (and $5'.6'$) of the path the X amount increases on both sides of the membrane.

System III (Table III).—In many respects this system resembles the preceding one, the following things namely appear from Table III. Between the determinations 7 and 8 the liquids get the same X amount; the X amount of the left side liquid becomes a maximum in the vicinity of the liquid marked \dagger (*viz.* 5.666 per cent NaCl); the X amount of the right side liquid becomes a minimum in the vicinity of the liquid, marked \dagger (*viz.* 5.260 per cent NaCl). For all that, there is a great difference between both systems. For in system II, first the right minimum occurs and afterwards the left maximum; during the osmosis in system III we have just the reverse, for here the left maximum occurs first and afterwards the right minimum. Consequently, system III may indeed be represented schematically by Figs. 4 and 5, if we interchange the places of maximum and minimum with respect to one another. The scheme of this system, which the reader may easily deduce himself, contains also symbols different from those in scheme II.

Besides the paths discussed above, we have determined those of other systems:

$$\text{Water} \mid L_1' \quad (19)$$

In Table IV the composition of the liquid L_1' of eight systems is found. The complete tables of the systems 1 (I), 4 (II) and 6 (III) are found in Tables I, II and III respectively; if any one should be interested in the other tables, I shall be pleased to forward them. The form of the path has been indicated under "type"; I means a form as in system I, etc. As in II and III, the systems 7 and 8 have a maximum in their left side path and a minimum in their right side path; as they occur, however, almost at the same time, it is difficult to say whether they belong to II or to III.

In these systems we have used two membranes H and W (no attention should be paid to the small letters a , b , etc. in Table IV): both consisted of a piece of pig's bladder; H , however, was of another bladder than bladder VI. It appears from Table IV that the paths of the eight systems (19) belong to three different types; consequently we put the question: what is the cause?

Of course the form of the path depends on the composition of liquid L_1' , consequently on the position of point $1'$ in the diagram. However it appears from the table that L_1' has almost the same composition in the systems 1 and 4: yet the type of both paths is different. We see the same thing for the systems 3 and 6 and also for 3 and 7. We have already seen that the form of the path depends on $n_1:n_1'$; this influence, however, has been eliminated here as much as possible.

As has been discussed above, the form of the path, however, depends also on the membrane. It now appears from Table IV that type I occurs in all systems (*viz.* 1–3) in which bladder H has been used and type II or III in all systems in which bladder VI has been used. Consequently this makes it probable that the cause of this phenomenon must be looked for in a difference between the bladders H and VI.

This shows that the nature of the membrane influences the form of the path; later on we shall see this confirmed by other examples. If we bear in mind that all sorts of influences (*e.g.* the action of the diffusing substances, age, hysteresis, etc.) may modify the nature of the membrane (permeability, adsorptive capacity, etc.) it becomes clear that, even when using apparently the same membrane, the type of the osmosis path may differ.

The question then arises in which direction do these substances X , Y and W diffuse through the membrane? Although we shall not discuss this question until a later communication I wish to emphasize the point that great care must be used. Let us take *e.g.* scheme II or Table II which obtains for system II. We see from them that the Y amount of the left side liquid increases perpetually whereas that of the right side liquid decreases. If we should deduce from this that during the osmosis Y (Na_2CO_3) diffuses perpetually towards the left, our conclusion would not be based on a single ground; it may be wrong as well as right.

This appears at once when we consider *e.g.* the change of the X

amount on part 5.6 of the path. We see that this now increases on both sides of the membrane. For the change in concentration of a substance not only depends on the direction in which this substance passes through the membrane, but also on the directions of the other substances. The X and Y amounts of the liquids change when nothing but water diffuses. (Cf. *e.g.* normal and abnormal changes in scheme 24 of Paper I.) Consequently from the vertical arrows no conclusion may be drawn with respect to the directions in which the substances pass through the membrane. As we shall see later on, we can only deduce them by considering the synchronous changes in concentration of the three substances.

THE EFFECT OF VALENCE OF IONS ON CELLULAR PERMEABILITY TO WATER.

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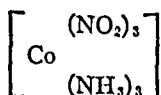
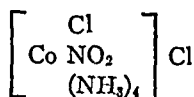
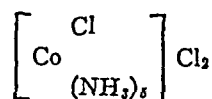
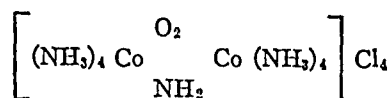
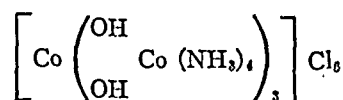
When a cell, such as the egg of the sea urchin, is placed in a solution hypotonic with respect to its natural medium, water enters under the driving force of osmotic pressure, and the cell swells. In the case of the spherical sea urchin egg the osmotic swelling proceeds relatively slowly and permits accurate measurements of the diameter, from which volume and surface of the cell can be calculated. The rate of entrance of water, per unit of time, per unit of surface and per unit of pressure may be defined as the permeability of the cell to water, and depends on a number of factors such as temperature and composition of the medium (1, 2).

While studying the effect of electrolytes on permeability of *Arbacia* eggs to water, striking differences were found in the effects of the chlorides of Ca and Mg on the one hand, and those of Na, K, Li and NH_4 on the other (2).^{*} In quite low concentration, chlorides of the bivalent cations decreased permeability to water while those of the univalent cations had the opposite effect. Since we were unable to find constant differences in the effects of cations of the same valence, it seemed likely that we were dealing with conditions in which effect of valence predominated over other ionic effects.

If this were the case, cations of valence greater than 2 should prove still more effective in reducing permeability to water. In fact preliminary experiments with LaCl_3 and ThCl_4 gave results pointing in this direction, but solutions of these salts were so acid that we regarded them as unsuitable and looked for a valence series of cations the salts of which would form neutral solutions.

^{*} Experiments dealing with Li and NH_4 are unpublished.

This requirement was met by a series of cobaltammine chlorides having the following structure:¹



This series has the advantages of containing cations of widely different valence ranging from 6 to 0; the compounds form approximately neutral solutions, and have been reported to undergo complete electrolytic dissociation as determined by conductivity measurements and other methods (3).^{*} The action of this cation series on permeability to water was studied by the following method.

Method.

Unfertilized eggs of *Arbacia punctulata* were washed in a solution of dextrose isotonic with sea water to eliminate electrolytes from the medium. The cells were then placed in a dish containing about 20 cc.

¹ The cobaltammine chlorides were kindly prepared for us by Professor Thomas P. McCutcheon, of the University of Pennsylvania. These compounds are representative of a larger number used in the experiments.

^{*} When *Arbacia* eggs were exposed in isotonic dextrose to concentrations of these salts such as were used in the experiments most of them remained capable of fertilization and development.

of 0.38 molal dextrose solution in which had been dissolved the salt to be tested. This solution is isotonic with 40 per cent sea water (40 parts of sea water and 60 parts of distilled water). The course of swelling at constant temperature ($12 \pm 0.5^\circ\text{C}.$) was then observed through the microscope for 4 to 6 minutes and the mean volumes of 5 to 10 cells were plotted against time. The rate of entrance of water in cubic micra per minute is given by the rate of increase in cell volume, $\frac{dV}{dt}$; it is obtained from the slope of the curve at a given time, t . The

permeability at this time is then $\frac{dV}{dt}/SP$, where S is the surface of the cell, and P is the difference in osmotic pressure between the interior of the cell at time t , and the medium.²

Effect of Valence of the Cation.

In the first group of experiments permeability was determined with a series of cobaltamine chlorides in 0.0001 molar concentration dissolved in hypotonic dextrose solution; the range of valence of the cation was from 1 to 6. It was found that salts with bivalent cations produced lower permeability than did those with univalent cations. But by this method, no significant difference in effect was found between the bivalent salts and those of higher valence.

The reason for this appears to be that, at a given temperature, permeability can be reduced only to a certain value. This numerical value is obtained in sea water (presumably on account of the presence of Ca and Mg) and also in dextrose solution containing cobaltamines of bivalent or higher valence in sufficient concentration.

It occurred to us that differences in effect of cobalts of higher valence might be demonstrated if we added some substance which increased permeability, and then determined the amount of cobalt necessary to reduce permeability to that obtained in sea water. This proved to be the case. It had been found that small amounts of K_2SO_4 and especially of K_3 citrate greatly increased permeability. When one of these

² For details of calculation see (2). In the present experiments slopes of curves were obtained by drawing tangents. Permeability varies but little during the course of an experiment; as a matter of convenience permeability was calculated at the 3rd minute.

TABLE I.

The effect of cobaltammine chlorides, having cations of different valence, on permeability to water. These salts in the concentrations shown are dissolved in 0.38 molal dextrose solution containing 0.01 M K_3 citrate. Solutions are isosmotic with 40 per cent sea water. Cell volumes are given in cubic micra $\times 10^2$. Each number represents the mean volume of 5 to 10 cells.

In the bottom row is given the permeability, which is the number of cubic micra of water entering the cell per minute, per square micron of surface, per atmosphere of pressure. The temperature was $12 \pm 0.5^\circ C$.

It is seen that the effectiveness of the cobaltammine chlorides in lowering permeability increases with the valence of the cation.

The mean volume of 20 control cells in sea water was 2000×10^2 cubic micra.

Molar concentration	6 valent		4 valent			3 valent			2 valent
	1×10^{-4}	2×10^{-4}	2×10^{-4}	4×10^{-4}	8×10^{-4}	4×10^{-4}	8×10^{-4}	1.6×10^{-3}	1.6×10^{-3}
<i>min.</i>									
1	2156	2139	2105	2205	2200	2187	2197	2139	2191
2	2308	2253	2280	2344	2313	2363	2313	2285	2423
3	2447	2326	2447	2442	2381	2636	2466	2377	2616
4	2631	2404	2611	2591	2452	2893	2626	2490	2834
5		2533		2721	2572		2778	2601	
Permeability	0.089	0.041	0.097	0.072	0.048	0.159	0.088	0.063	0.131

TABLE II.

The effect of valence in a cation series of cobaltammine chlorides. The salts in the concentrations shown are dissolved in 0.38 molal dextrose solution containing 0.005 M K_3 citrate. Numbers represent permeability to water.

The effectiveness of the cation in reducing permeability to water increases with the valence; for example, 5×10^{-5} of the 6 valent salt are required to reduce permeability to the value usually obtained in sea water, while 64 times this concentration is required in the case of the 2 valent cobalt (data are graphed in Fig. 2).

Molar concentration	2.5×10^{-5}	5×10^{-5}	1×10^{-4}	2×10^{-4}	4×10^{-4}	8×10^{-4}	1.6×10^{-3}	3.2×10^{-3}
6 valent	0.194	0.048	0.042					
4 valent		0.172	0.058	0.038				
3 valent			0.180	0.068	0.061			
2 valent					0.171	0.113	0.157	0.043
1 valent								0.058

salts in proper concentration was added to the solution it was found that much less of 6 valent cobalt was required to reduce permeability than of the 4 valent, and so on down the series. Thus in Table I it is seen that when 0.01 M K_3 citrate was used, 0.0002 M 6 valent cobalt was sufficient to reduce permeability to the value obtained in sea water,* 0.0008 M of the 4 valent was required, while twice this amount of the 3 valent and 2 valent salts were incompletely effective.

When a lower concentration of K_3 citrate (0.005 M) was used, smaller amounts of the cobalts were necessary to reduce permeability. In Table II it is seen that 0.00005 M of the 6 valent salt was sufficient to reduce permeability, more than twice as much of the 4 valent cobalt was required, more than 8 times as much 3 valent and 64 times as much of the 2 valent salt, while this amount of the 1 valent salt was incompletely effective.

Definite though less striking differences in effectiveness were found between several bivalent and univalent cobaltammines, the former decreasing permeability to water, whereas univalent cobaltammines, as well as the undissociated compound, did not decisively either increase or decrease permeability.³

From these and similar experiments it is concluded that there are distinct differences in the effectiveness of cobaltammines of different valence in reducing permeability and that this effectiveness increases rapidly with the valence of the cation.

Effect of Valence of the Anion.

The results obtained with the cobalt cation series suggested the question, do anions act in the opposite direction? Do they tend to increase permeability, and is their effectiveness related to valence?

Preliminary experiments indicated that this is the case. When 0.0001 M KCl, K_2SO_4 , K_3 citrate and $K_4Fe(CN)_6$ were added to dextrose solution, sulfate gave higher permeability than chloride, while citrate and ferrocyanide gave higher permeability than did sulfate.

* In 40 per cent sea water, or in 0.38 molal dextrose solution containing 0.0005 M $CaCl_2$, the value for permeability at 12°C. is approximately 0.05, but varies somewhat in different lots of cells.

³ When cobaltamine appears as a univalent anion, $\left[\begin{array}{c} Co \quad (NO_2)_4 \\ (NH_3)_2 \end{array} \right] Na$, definite increase in permeability was demonstrable.

However with such solutions, tendency to cytolysis was a disturbing factor; this was overcome by addition of CaCl_2 in 0.0005 molar concentration. It was now possible to determine the minimal concentration of the potassium salt which would increase permeability above the magnitude obtained in sea water or in CaCl_2 solution. Two series of potassium salts were used, one with inorganic, one with organic anions.

TABLE III.

The effect of valence in an anion series of inorganic potassium salts. The salts in the concentrations shown are dissolved in 0.38 molal dextrose solution containing 0.0005 M CaCl_2 . Cell volumes measured at minute intervals are given in cubic micra $\times 10^2$. Each number represents the mean volume of 6 to 8 cells.

In the bottom row is given the permeability in the units stated in Table I. The temperature was $12 \pm 0.5^\circ\text{C}$.

It is seen that the effectiveness of the salts in raising permeability above the value of the CaCl_2 control increases with the valence of the anion.

The mean volume of 20 control cells in sea water was 2236×10^2 cubic micra.

Molar concentration	$\text{K}_4\text{Fe}(\text{CN})_6$	$\text{K}_3\text{Fe}(\text{CN})_6$			K_2SO_4		KCl		CaCl_2
	1×10^{-3}	1×10^{-3}	2×10^{-3}	4×10^{-3}	2×10^{-3}	4×10^{-3}	4×10^{-3}	8×10^{-3}	
<i>min.</i>									
1	2428	2423	2418	2405	2405	2433	2414	2368	2428
2	2616	2523		2626	2499	2676	2494	2509	2567
3	2861	2656	2676	2824	2616	2882	2567	2641	2671
4	3059	2757	2840	3032	2742	3154	2676	2793	2742
5	3290	2871	3026		2876		2783	2935	2824
6	3478	3005	3199		3005		2887	3059	2924
Permeability	0.118	0.057	0.081	0.115	0.062	0.133	0.046	0.073	0.048

With the former, Table III illustrating a typical experiment, shows that ferrocyanide was more effective in increasing permeability than 8 times the amount of chloride and had approximately the same effect as 4 times the amount of sulfate, while sulfate was more than twice as effective as the chloride.

The question might be raised, however, whether this result could be due to differences in concentration of potassium ions rather than to valence of the anion. That this is probably not the correct or at least the complete explanation is evident from the ratios of the effective concentrations as given in the preceding paragraph.

Table IV brings out the effect of valence still more clearly. In this experiment 0.001 M ferrocyanide was required definitely to increase permeability above the value of the control (CaCl_2 solution), twice as much ferricyanide, 4 times as much sulfate and 8 times as much chloride.

TABLE IV.

The effect of valence in an anion series of inorganic potassium salts. The salts in the concentrations shown are dissolved in 0.38 molal dextrose solution containing 0.0005 M CaCl_2 . Numbers represent permeability to water.

The effectiveness of the salts in increasing permeability above the value of the CaCl_2 control increases with valence of the anion.

Molar concentration	2×10^{-4}	8×10^{-4}	1×10^{-3}	2×10^{-3}	4×10^{-3}	8×10^{-3}
KCl				0.066	0.067	0.095
K_2SO_4				0.048	0.152	
$\text{K}_2\text{Fe}(\text{CN})_6$			0.068	0.107		
$\text{K}_4\text{Fe}(\text{CN})_6$	0.056	0.063	0.087	0.192		

($\text{CaCl}_2 = 0.046$)

TABLE V.

The effect of valence in an anion series of organic potassium salts.

The effectiveness of the salts in increasing permeability above the value of the CaCl_2 control increases rapidly with the valence of the anion (data are graphed in Fig. 1).

Molar concentration	2.5×10^{-4}	5×10^{-4}	1×10^{-3}	2×10^{-3}	4×10^{-3}	8×10^{-3}
K acetate			0.044	0.049	0.048	0.116
K_2 tartrate		0.055	0.060	0.090	0.150	
K_3 citrate	0.052	0.086	0.184			

($\text{CaCl}_2 = 0.046$)

Similar results were obtained using a series of organic salts with increasing valence of the anion (Table V): 0.0005 molar citrate was required to increase permeability above the value of the CaCl_2 control; 4 times as much tartrate and 16 times as much acetate were needed.

In both inorganic and organic anion series it was invariably found that the effectiveness of the salts in increasing permeability to water increases rapidly with the valence of the anion.

The relation between permeability and valence in the anion and cation series is graphically shown in Figs. 1 and 2. In the first graph (Fig. 1) permeability to water is plotted against the molar concentration of an anion series. It is seen that with the trivalent anion a relatively slight increase in concentration causes a marked change in

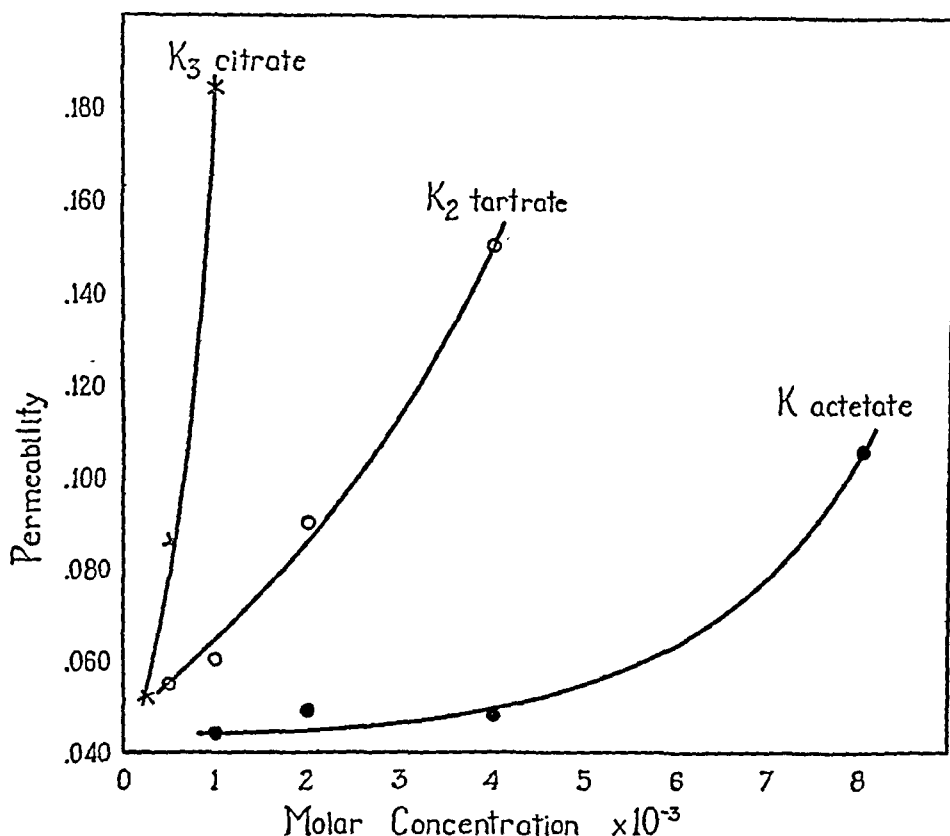


Fig. 1. The relation between permeability and molar concentration of salts having anions of different valence (data are taken from Table V).

permeability; whereas with the univalent anion a considerable change in concentration is required to bring about an increase in permeability; and the bivalent anion occupies an intermediate position.

Similarly a plot of a cation series (Fig. 2) shows that permeability to water decreases with concentration of the 6 valent cation more rapidly than with cations of lower valence.

DISCUSSION.

The experiments indicate that permeability of cells to water is regulated, at least in part, by the sign and the number of charges on the ions in the medium, in the sense that cations decrease and anions increase permeability to water; and these effects increase rapidly with the valence of the ion.

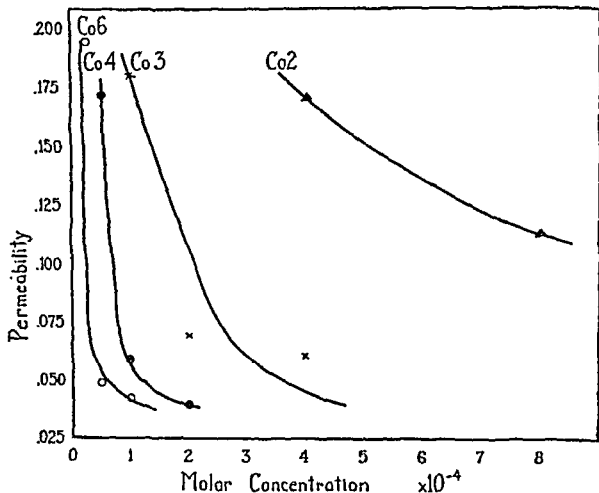


Fig. 2. The relation between permeability and molar concentration of salts having cations of different valence (data are taken from Table II).

It is interesting to note that valence and the sign of the charge were found by Osterhout (4) and Raber (5) to produce changes in conductivity of plant tissues that are closely paralleled in our experiments on permeability to water.

The experiments also show that it is possible to determine the concentration of a cation which will oppose the effect of an anion in its tendency to increase permeability to water, and conversely, the

restraining action of a cation on osmosis may be balanced by the proper concentration of an anion. The method used, therefore permits quantitative study of antagonism between cations and anions in their effects on permeability to water.

SUMMARY.

1. Permeability to water in unfertilized eggs of the sea urchin, *Arbacia punctulata*, was studied by measuring the rate of swelling in hypotonic dextrose solution.

2. Permeability is greatly affected by addition of electrolytes in low concentration.

3. A decrease in permeability to water was found with increasing valence of the cation, using a series of cobaltammine chlorides in which the valence of the cation ranged from 1 to 6.

4. Conversely, an increase in permeability to water was found with increasing valence of the anion, using two series of potassium salts in which the valence of the anion ranged from 1 to 4, and 1 to 3, respectively.

5. It is concluded that the effect of electrolytes on permeability to water depends chiefly on the sign and the number of charges on the ion, in the sense that positive ions decrease permeability to water, while negative ions increase permeability to water; and the effectiveness of the ion is greater the higher its valence.

6. Antagonism has been demonstrated between cations and anions in their effect on permeability, and the method employed permits quantitative study of such antagonism.

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ON PYRIDINE HEMOCHROMOGEN.

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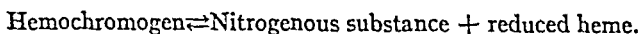
(Accepted for publication, January 2, 1929.)

The writers (1, 2) have shown that every hemochromogen consists of reduced heme (the iron pyrrol complex of hemoglobin) combined with some nitrogenous substance. They have furthermore given evidence (2) that in cyanide hemochromogen there is one cyanide group for every heme group. There had previously been two attempts to determine the composition of pyridine hemochromogen. Von Zeynek (5), before the nature of hemochromogen was understood, found solid pyridine hemochromogen to contain 2.2 molecules of pyridine per heme. He regarded his single experiment as only preliminary and gave no certain evidence that the solid analyzed was pure pyridine hemochromogen. Recently R. Hill (3) concluded that pyridine hemochromogen contains two pyridines per heme. This conclusion is hardly justified by Hill's experiments. And Hill did not consider two important difficulties, one concerned with the precipitation of reduced heme, the other with the aggregation of pyridine hemochromogen. We propose to discuss in some detail the difficulties in the study of the pyridine hemochromogen equilibrium for they are of general interest in the chemistry of the hemochromogens. The complications of the pyridine reactions will be easier to understand if we first review the simpler reactions with cyanide.

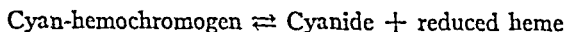
The Reactions between Cyanide and Reduced Heme.

One molecule of KCN per molecule of reduced heme and not less suffices to convert a 2×10^{-4} M solution of reduced heme into cyan-hemochromogen. This proves that cyan-hemochromogen does not contain more than one cyanide group per heme. It does not exclude the possibility that cyan-hemochromogen contains less than one cy-

anide group per heme. For we have shown (1,2) that in every hemochromogen there is the equilibrium:



So some of the cyanide added must be free to drive the equilibrium



to the left. The following considerations, however, indicate that the free cyanide in the 2×10^{-4} M solution of cyan-hemochromogen is negligibly small.

In a mixture of reduced heme and hemochromogen the fraction of the total pigment which has to be in the form of reduced heme in order for the spectrum of reduced heme to be seen is independent of the total pigment concentration provided the more dilute the solution the greater the thickness of it through which one looks. Let us assume that the concentration of free cyanide needed to prevent the appearance of reduced heme, that is, to convert a given fraction of the pigment into hemochromogen, is independent of the total pigment concentration in concentrations less than 2×10^{-4} M. On this hypothesis as the total concentration of pigment is made smaller, the bound cyanide becomes smaller while the free cyanide remains the same. In a very dilute solution, then, the free cyanide is approximately equal to the total cyanide added. We found that in such a dilute solution it takes 3.75×10^{-5} M cyanide to prevent the appearance of reduced heme. If the free cyanide is likewise 3.75×10^{-5} M in the relatively concentrated 2×10^{-4} M solution where it takes only 1 molecule of KCN to convert a molecule of reduced heme into hemochromogen, then in this case the free cyanide is within the experimental error negligible and so cyan-hemochromogen probably does not contain less than one cyanide group per heme.

The Reactions between Pyridine and Reduced Heme.

In his study of the composition of hemochromogen R. Hill (3) used pyridine as his nitrogenous substance. We had pointed out (1) that of the substances originally tested pyridine and nicotine had the greatest affinity for reduced heme. Later experiments, however, showed that cyanide has an affinity for reduced heme some fifty times

greater than that of pyridine. Hence the use of cyanide in the experiments just described and the avoidance so far as possible of some of the serious difficulties which will now be discussed.

Hill determined the number of pyridine molecules per heme needed to prevent the appearance of reduced heme in solutions of various concentrations. He states that reduced heme can be detected if present as 5 per cent of the total pigment. Columns 1 to 3 of Table I give Hill's results. The other columns give the calculations omitted by Hill which we based on his results.

In the most dilute solution (0.62×10^{-3} M) the total pyridine was 2.34×10^{-3} M. If one pyridine was bound per heme the free pyridine was $(2.34 - 0.62) \times 10^{-3}$ M or 1.72×10^{-3} M. If two pyridines were

TABLE I.

Total heme M $\times 10^3$	Total pyridine M $\times 10^3$	Total pyridine Total heme	Free pyridine = 1.72×10^{-3} M		Free pyridine = 1.10×10^{-3} M	
			Bound pyridine M $\times 10^3$	Pyridine bound per heme	Bound pyridine M $\times 10^3$	Pyridine bound per heme
7.69	14.6	1.9	12.9	1.68	13.5	1.76
3.42	6.50	1.9	4.78	1.40	5.40	1.58
2.20	4.62	2.1	2.90	1.32	3.52	1.60
1.62	3.56	2.2	1.84	1.14	2.46	1.52
1.18	2.83	2.4	1.11	.94	1.73	1.46
.62	2.34	3.8	.62	1.00	1.24	2.00

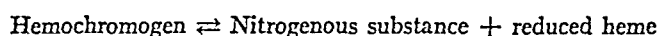
bound the free pyridine was $(2.34 - 2 \times 0.62) \times 10^{-3}$ M or 1.10×10^{-3} M. Let us assume, since some assumption is necessary, that the free pyridine was the same in all the solutions. The bound pyridine in the more concentrated solutions may now be obtained by subtracting the hypothetically constant free pyridine from the total pyridine added. Finally the values of pyridine bound per heme are calculated by dividing the bound pyridine by the total heme. These values are inconsistent and all less than two.

Hill's conclusion that pyridine hemochromogen contains two pyridine groups per heme thus rests on the unproven assumption that the free pyridine was negligible in his most concentrated solutions. If the free pyridine was the same in all the solutions then this assumption is incorrect. Furthermore, we shall now present evidence which indi-

cates that the free pyridine in the concentrated solution was actually greater than in the dilute solutions, that the corrections for the free pyridine should be greater than they are in Table I rather than less as is necessary to justify Hill's conclusion.

The Precipitation of Reduced Heme.—Cyanide has an affinity for reduced heme some fifty times greater than that of pyridine. In order, therefore, for the free nitrogenous substance to be negligible compared with the bound a solution of pyridine hemochromogen must be some fifty times as concentrated as a similar solution of cyan-hemochromogen. Hill's solutions, it has been pointed out, were not concentrated enough for the free pyridine to be negligible. But they were concentrated enough to introduce the problem of the precipitation of reduced heme.

Let us suppose that in the equilibrium mixture



90 per cent of the pigment is in the form of hemochromogen. If the total concentration of pigment is low enough the 10 per cent of reduced heme formed on adding the reducing agent will remain in solution. This small percentage of reduced heme cannot be detected spectroscopically. If, however, the total concentration of pigment is high enough, the amount of the sparsely soluble reduced heme formed will be greater than the amount which can stay in solution. Reduced heme will precipitate, more hemochromogen will dissociate to maintain the equilibrium and so on. Under suitable conditions the continued formation of reduced heme can readily be followed with the spectroscope. Finally, the concentration of reduced heme in solution will correspond to the solubility of reduced heme or at least to some condition where reduced heme does not precipitate rapidly. But the total reduced heme in solution and a precipitate will now be more than 10 per cent of the total pigment and the spectrum of reduced heme will be seen.

It follows from these considerations that to prevent the precipitation and hence the appearance of reduced heme, it is sufficient in the dilute solution to convert 90 per cent of the pigment into hemochromogen leaving 10 per cent as reduced heme. In the concentrated solution, however, one cannot have in solution as reduced heme so much as 10

per cent of the total pigment and so more than 90 per cent must be converted into hemochromogen. This can be done only by increasing the concentration of free nitrogenous compound. To prevent, therefore, the appearance of reduced heme one must have a higher concentration of free nitrogenous compound in the concentrated solution than in the dilute solution.

As shown in Table I 1.9 pyridine molecules per heme have to be added to a 7.69×10^{-3} M solution of reduced heme in order to cause the disappearance of the reduced heme spectrum. If the free pyridine here is 1.72×10^{-3} M as in the more dilute solution, then 1.68 pyridines are bound per heme. But in the concentrated solution of pyridine hemochromogen the difficulty concerning the precipitation of reduced heme enters in to an unknown extent. The free pyridine needed to prevent the appearance of reduced heme would on this basis be higher than the 1.72×10^{-3} M needed in the dilute solution and fewer than 1.68 pyridines would be bound per heme. Hill did not take this difficulty into account.

The Aggregation of Pyridine Hemochromogen.—The second difficulty in the study of the pyridine hemochromogen equilibrium is concerned with the possible aggregation of the hemochromogen itself. Pyridine hemochromogen in a 10 per cent pyridine solution has a typical sharp narrow hemochromogen α band, which is about 25 Å. u. toward the blue of the α band of globin hemochromogen. If the solution contains only 0.004 M pyridine and just enough pigment so that one can readily see the hemochromogen band when looking through a layer 2 cc. thick, then immediately on reduction the α band is in the same position as in the 10 per cent pyridine solution. But within a few seconds there is a shift of the band toward the red of about 30 Å. u. and within a few minutes a further shift of about 10 Å. u. The band, furthermore, becomes broader and asymmetrical, the part toward the red being darker than the part toward the blue. On half saturation with $(\text{NH}_4)_2\text{SO}_4$ the band shifts still further toward the red and becomes still more asymmetrical. All this is in harmony with our earlier observations (1) and with those of Keilin (4).

In our original paper (1) we showed that such shifts in the position of a hemochromogen band are usually accompanied by a precipitation of the hemochromogen. Keilin (4) has suggested that it is the change

in the dispersion of the pigment to which the change in the spectrum is directly due. This correlation between dispersion and spectrum is most easily observed with globin hemochromogen. If this hemochromogen is precipitated by salting out in alkaline solution, by neutralization, or by dilution of a neutral concentrated urea solution then the band shifts some 25 Å. u. toward the red.

The observations on pyridine hemochromogen therefore indicate that when the solution is dilute in respect to pyridine the hemochromogen is not molecularly dispersed. Hill did not recognize this difficulty nor is it clear how this difficulty even when recognized can be taken into account in the calculations.

CONCLUSIONS.

1. Cyanide hemochromogen probably contains one cyanide group per heme group.
2. The equilibrium between pyridine hemochromogen and its components, pyridine and reduced heme, is complicated to an unknown extent by the precipitation of reduced heme and the aggregation of pyridine hemochromogen.
3. These complications were not taken into account in R. Hill's experiments on pyridine hemochromogen.
4. Even if Hill's experiments are sound they do not prove his conclusion that pyridine hemochromogen contains two pyridine groups per heme group.

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THE INFLUENCE OF SIZE, SHAPE AND CONDUCTIVITY OF MICROSCOPICALLY VISIBLE PARTICLES ON CATAPHORETIC MOBILITY.

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INTRODUCTION.

Although the cataphoresis of microscopically and submicroscopically visible particles has been very frequently investigated, the influence of size, shape and conductivity of the particle has not been completely determined. Solution of this problem is of the utmost importance for all applications of the method of cataphoresis. Before differences in mobility in an electric field may be ascribed to variations in the nature of the electric conditions at the phase boundary of the particle, the influence of variation of size, shape and conductivity of particles equal in other respects must be recognized. The theories of Helmholtz, Lamb, and von Smoluchowski (1) lead to the formula for cataphoresis.

$$V = \frac{1}{4\pi} \frac{X D \xi}{\eta},$$

(V = cataphoretic velocity. X = field strength. D = dielectric constant of the medium. ξ = electrokinetic potential. η = viscosity of the medium. All units c.g.s. electrostatic.) According to this formula neither size nor shape should influence the migration velocity. Debye and Hückel (2), making certain assumptions, came recently to the conclusion that the factor $\frac{1}{4\pi}$ was valid only for cylindrical particles whereas the factor $\frac{1}{6\pi}$ should be substituted in the case of spherical particles. This difference is great enough to be determined experimentally.

Previous experiments dealing with the effect of size, shape and conductivity may be briefly summarized as follows. Hardy (3) found that various solutions of submicroscopic particles of protein prepared by adding different amounts of acetic acid to the protein solutions had identical cataphoretic mobilities. As the different solutions varied in diffraction color, it was believed that the mobility observed was independent of particle size. Burton (4) found that Bredig silver solutions presumably of different particle size had equal cataphoretic velocities. Ellis (5), however, referring to a statement of Cotton and Mouton that the velocity varies with size, compared droplets of the same size only. McTaggart (6) noted that gas bubbles 60μ to 160μ in diameter migrated independently of the diameter. In contrast to these findings are the experiments of Mooney (7). He found that for droplets of Nujol* 0.5μ to 40μ in diameter, there was a considerable increase in migration velocity with increasing diameters of the droplets. Thus for a 22 fold increase in diameter, from 0.5μ to 11μ , the velocity was doubled. Mooney obtained similar results for ten other kinds of emulsions made up in water and in dilute electrolytes. In CuSO_4 solutions these differences supposed by Mooney to depend upon particle size disappeared. The technic employed by Mooney was different from that used by most recent authors. He worked with a vertical, cylindrical, capillary glass tube with sealed in electrodes. The effect of gravity on the droplets, although presumably accounted for in estimation of velocity, was considerable. These observations of Mooney are unique in that such marked differences in velocity had never previously been described. Freundlich and Abramson (8) and Abramson (9) found that (1) single red blood cells and their aggregates of different sizes and shapes, (2) irregularly shaped polymorphonuclear leucocytes, (3) quartz particles of various sizes (0.5μ to 15μ) and shapes in water, dilute electrolytes and non-electrolytes, (4) glass and clay particles suspended in similar solutions, all migrated independently of size and shape. McTaggart's experiments with air bubbles were also confirmed by studying air bubbles in gelatin gels.

Although there is no extensive theoretical treatment of the influence of particle conductivity on cataphoretic mobility, the theories have always been derived for insulators.

* A paraffin oil.

In connection with the influence of the conductivity of the particles two problems arise for discussion. First, the whole particle may be homogeneous. In this case the influence of the conductivity of the particle cannot be investigated experimentally as the conductivity of the particle cannot be varied without varying the composition of the whole particle including its phase boundary. Secondly, the particle may be heterogeneous when the surface film is composed of material entirely different from that of the bulk. Particles having identical surface films, but varying in the conductivity and chemical make-up of the enclosed bulk of the particles may be then compared. Such particles can be obtained by suspending particles of varied chemical nature and electric conductivity in dilute protein solutions (10). The adsorbed film of protein determines the properties of the surface which is responsible for the mobility of the particle, while the chief material is excluded from direct contact with the medium. If the material making up the bulk of the particle were to influence the velocity of migration, then this influence could only be due to the varying conductivity if the influence of size and shape of particle can be excluded.

Methods.

Apparatus.—A horizontally fixed microscopic cataphoresis cell similar in arrangement to that described by Northrop and Kunitz (11) was employed. Certain modifications were found useful which have been described in detail elsewhere (12). The cell was of Pyrex glass, about 0.8 mm. in depth, 9 mm. wide, and the hydrodynamic conditions followed the principles laid down for such systems by von Smoluchowski (1). Non-polarizable electrodes were used. The applied E.M.F.'s were great enough to give easily measurable velocities with no heating effects.

Materials.—Droplets of Nujol, benzyl alcohol, castor oil, a paraffin oil, and coca butter were studied in media (alcohol-water, cane sugar-water mixtures containing traces of electrolytes) of approximately the same specific gravity as the oils themselves. Certain droplets were studied in systems containing sufficient protein to give the droplets at least a complete surface of protein. This procedure gives very uniform surfaces because of the similarly adsorbed protein films, and was

TABLE I.

Nujol in Water-Ethyl Alcohol Mixture Having Same Density as the Oil. A Few Drops of pH 7.4 Phosphate Buffer Added to About 50 cc. of Emulsion. The Same Result Was Obtained for Emulsions in Methyl Alcohol Mixtures.

Diameter of droplet	Time to move 200 μ	Diameter of droplet	Time to move 200 μ
μ	sec.	μ	sec.
30	15.2	0.5	14.5
1	15.3	13	14.5
5	15.2	13	14.6
3	14.6	5	14.4
13	14.7	1	14.4

TABLE II.

Benzyl Alcohol + Small Amount of Sugar + Few Drops pH 7.4 Buffer.

Diameter of droplet	Time to move 300 μ	Diameter of droplet	Time to move 300 μ
μ	sec.	μ	sec.
45	13.5	20	14.0
5	14.2	20	13.8
2	14.2	2	14.4
25	14.2	15	14.0
2	14.2	3	13.8
1	14.0	1	13.6

TABLE III.

Paraffin Oil in Ethyl Alcohol-Water Mixture + Few Drops pH 7.4 Phosphate Buffer.

Diameter of droplet	Time to move 100 μ	Diameter of droplet	Time to move 100 μ
μ	sec.	μ	sec.
{ 25	9.8	{ 20	8.4
{ 2	9.0	{ 2	8.2
{ 5	8.8		
{ 20	9.4	{ 2	6.0
		{ 15	6.2
{ 15	12.0		
{ 3	12.2		

The brackets are groups from different levels in the cataphoresis cell.

TABLE IV.

Coca Butter.

<i>Experiment (a). In distilled water without protein film</i>			<i>Experiment (b). The suspension was here in 0.05 per cent gelatin + dilute HCl (about N/1000)</i>		
Diameter of droplet	Time to move same distance	Remarks	Diameter of droplet	Time to move same distance	Remarks
μ	sec.		μ	sec.	
8	5.2	Note a greater variation than in part (b)	8	4.6	The speed of the droplets is more uniform and independent of the size with the protein film
5	5.4		1	4.2	
3	6.2		4	4.4	
2	6.0		4	4.6	
2	6.0		2	4.6	
4	5.2		7	4.4	
7	5.4		5	4.6	
2	5.6		1	4.4	
			5	4.4	
			1	4.4	

TABLE V.

Castor Oil in Distilled Water + Few Drops of pH 7.4 Phosphate Buffer.

Diameter of droplet	Time to move 200 μ	Diameter of droplet	Time to move 200 μ
μ	sec.	μ	sec.
2	5.0	1	5.4
3	5.2	7	5.2
2	5.0	1	5.4
1	5.2	10	5.5
1	5.4	5	5.5

therefore used to study needles and droplets simultaneously. Asbestos needles of various lengths remained fairly well suspended in watery and dilute protein solutions, as did recrystallized *m*-aminobenzoic acid crystalline needles. In all experiments the following precautions were observed:

1. The vertical movements of the particles due to gravity were eliminated as far as possible, by having the density of the medium and of the particle similar.

2. Measurements were always made at the stationary water layer in the cell to make sure that the mobility observed in the mid-regions

(where most observations reported have been made) was not simply due to electroendosmotic streaming of the water within the cell.

EXPERIMENTAL.

1. *The Effect of Particle Diameter on Cataphoretic Mobility.*—Droplets of the following substances were studied: The details are in the tables where typical protocols are presented.

Nujol $0.5\ \mu$ to $35\ \mu$ Table I.

Benzyl alcohol $0.5\ \mu$ to $45\ \mu$ Table II.

Paraffin oil $2\ \mu$ to $25\ \mu$ Table III.

Coca butter $1\ \mu$ to $8\ \mu$ Table IV.

Castor oil $1\ \mu$ to $10\ \mu$ Table V.

All our experiments have been performed in the presence of sufficient electrolyte or protein to avoid the complications found in Mooney's experiments. As mentioned previously, this author noted that in electrolyte-poor emulsions, droplets having larger diameters move more rapidly than smaller droplets. Mooney also noted that this difference in velocity was less in the presence of electrolytes. The observations of Mooney have been confirmed by the authors in very electrolyte-poor emulsions containing no proteins on a few occasions.

An analysis of the five tables just mentioned discloses that under the stated conditions *the cataphoretic velocity of oil droplets is independent of their diameter*. The larger drops were easily studied and as has been described under methods, media of almost identical specific gravity with that of the droplets were used. Very rarely (less than about $\frac{1}{5}$ of 1 per cent of all observations) anomalous behavior of droplets was observed. A droplet would migrate very quickly or very slowly. The relatively large changes produced by small amounts of impurities in the system is sufficient to account for these variations which are in no way related to their size or shape.

2. *The Effect of Particle Length on Cataphoretic Velocity.*—Asbestos pulp in dilute suspensions gives needles of easily studied lengths from $3\ \mu$ to $200\ \mu$. The diameter of the needles was from about $1\ \mu$ to $3\ \mu$. Long cylinders of very small radius were, therefore, available for study. These needles were studied suspended in distilled water and in dilute

protein solutions. Fig. 1 gives the data for these two types of media. In the figure the orientation of the particle is given by its position, the length of the particle by the scale and the time in seconds taken for the particles to migrate a given distance, the same for all particles, by

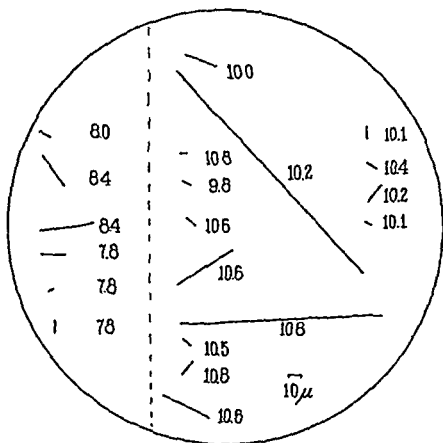


FIG. 1. The field is divided into two parts. In right lower hand corner is the scale to which the asbestos needles have been drawn. The slight differences in radius were neglected. The group of needles to the left of the dotted line were studied in distilled water with the orientation as when observed; the number to the right of each needle is the time in seconds required to migrate a given distance which was the same for the whole group. The needles to the right of the dotted line were studied in $N/1000$ HCl containing 0.05 per cent gelatin which coats the needles with a protein film. The numbers have the previous significance. This figure demonstrates that neither size nor orientation of particle changes the cataphoretic velocity.

the number next to the particle. It is evident that for such particles the cataphoretic velocity is independent of particle size, thus confirming with particles of entirely different shape the results obtained with oil droplets.

Orientation of Needle-Shaped Particles during Migration.

Most of the needle-shaped particles long enough to have no brownian movement are found in horizontal planes, but oriented at random within these planes. During the migration this random orientation is not disturbed over distance of 500μ or so.¹ These observations demonstrate that the streaming of fluid within the cell is practically laminar, making possible the application of von Smoluchowski's formula, indicating the relationship of the velocity of a particle to its depth within the cell.

The Effect of the Shape of the Particle on Cataphoretic Velocity.

The following needle-shaped particles were investigated:

(a) Asbestos needles from just visible lengths up to 70μ long.

(b) *m*-Aminobenzoic acid crystals (needles up to 100μ long).

These were compared in the same medium (protein solutions) with small paraffin oil or mastix globules 1μ to 5μ in diameter. When such substances are suspended in protein-free media they have different cataphoretic velocities because of their chemically different surfaces. When suspended in dilute protein (*e.g.* 0.1 per cent gelatin) solutions they adsorb a protein film and in this way, the needles and oil globules are given chemically similar surfaces. That the protein film in such instances may give values of cataphoretic velocity remarkably close to the native protein has been shown by Abramson (9) and by Freundlich and Abramson (8). These experiments have been confirmed by Briggs (13) using an entirely different method.

Fig. 2 shows groups of particles studied at the same level. The particles are drawn to the scale indicated or are of the size noted. An oil droplet and a needle were always studied at the same level at the same time. The time of migration over a given distance is given in seconds next to the particle. These experiments demonstrate that *for very extreme instances of particle size and shape the velocity of cataphoretic migration is here determined solely by the surface characteristics of the particle and is independent of both size and shape within the limits studied.*

¹ Red cell rouleaux have also been observed to exhibit the same phenomenon in serum and other liquids. The same was true for gelatin gels (8, 9). See also Abramson, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxvi, 147.

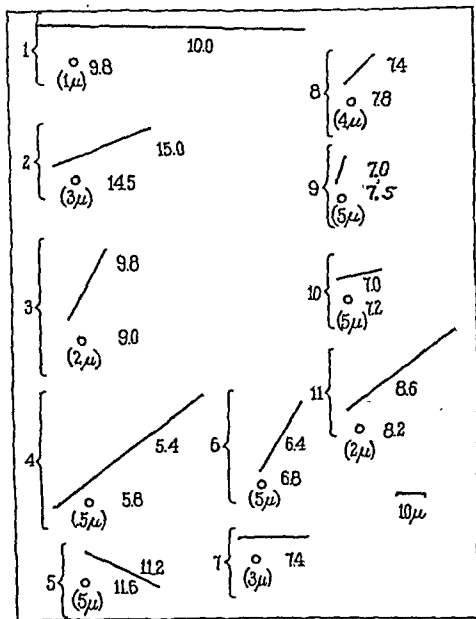


FIG. 2. The brackets indicate needles and globules studied in pairs. Typical experiments, Nos. 1-3, are *m*-aminobenzoic acid crystals and mastic globules covered with gelatin. Nos. 4-10 are asbestos needles and paraffin oil globules covered with albumin. No. 11 is a *m*-aminobenzoic acid crystal and a paraffin oil globule covered with gelatin. The oil droplets and mastic particles are all drawn the same size but their diameter is given in parentheses near the particle. The needles are drawn to the scale in the lower right hand corner.

Each pair was studied at the same level in the cell. The numbers following each particle are the relative speeds. It is evident that there is no change in cataphoretic velocity even for these limiting cases.

TABLE VI.

The Migration Velocity of Quartz and Benzyl Alcohol Droplets in Approximately N/100 and N/1000 HCl When Both Quartz and Benzyl Alcohol Have Similar Adsorbed Gelatin Films (Initial Concentration Gelatin 0.2 Per Cent).

N/100 HCl		N/1000 HCl		N/100 HCl		N/1000 HCl	
Time to migrate given distance		Time to migrate given distance		Time to migrate given distance		Time to migrate given distance	
	sec.		sec.		sec.		sec.
B	9.4	Q	5.8	B	9.5	Q	6.2
Q	9.0	Q	6.0	Q	10.2	B	6.0
Q	9.4	B	5.8	Q	9.2	B	5.8
B	9.0	Q	6.2	Q	9.4	Q	6.2
Q	8.8	Q	5.8	Q	9.4	Q	5.8
Q	9.6	B	6.0	B	10.0	Q	5.4
B	9.2	Q	5.6	Q	9.8	Q	5.8
Q	9.4	Q	6.0	Q	9.6	Q	5.5
B	9.1	B	5.8	Q	9.5		

B = benzyl alcohol droplet. Q = quartz particle.

TABLE VII.

Agar Particles in Equilibrium with N/50 Acetate Buffer, pH = 3.6, in $\frac{1}{2}$ Per Cent Gelatin Solution Migrate with the Same Speed as Paraffin Oil Droplets.

Time to migrate given distance		Time to migrate given distance	
	sec.		sec.
P	6.4	A	6.4
A	6.4	A	6.2
A	6.4	A	6.0
A	6.6	P	6.2
P	6.7	P	6.0
A	7.0	P	6.0
A	6.8	A	6.8
		A	6.0

The Same Experiment with Hemocyanine, pH = 4.0

	sec.		sec.
P	7.0	P	7.8
A	7.4	A	7.0
A	6.5	A	7.0
A	6.4		
A	6.8	P	9.0
P	7.2	A	8.4
A	7.0		
A	7.0		

Brackets refer to droplets studied at particular levels in the cataphoresis cell.
P = oil. A = agar.

The Influence of Particle Conductivity.

The following kinds of particles, all covered with protein films, were studied simultaneously in dilute electrolyte solutions: paraffin droplets, quartz particles, droplets of benzyl alcohol, carbon particles, particles of agar. The conductivity of the material of these particles varies from virtually zero in the case of quartz, up to a conductivity practically equal to that of the surrounding medium in the case of agar. The protein film was obtained either with gelatin or with

TABLE VIII.

Carbon Particles Suspended in N/100 HCl + $\frac{1}{2}$ Per Cent Gelatin Move with the Same Speed As Paraffin Oil Droplets.

Time to migrate given distance		Time to migrate given distance		Remarks
	sec.		sec.	
{P	6.0	{P	5.8	Brackets refer to particles studied at different levels in cataphoresis cell
{C	6.0	{C	6.0	
{P	5.8	{P	9.0	
{C	5.8	{C	9.4	
{P	4.5	{P	8.8	
{C	4.8	{C	9.4	
{P	6.6	{P	5.6	
{C	7.0	{C	5.4	

P = oil. C = Carbon.

hemocyanine (*Limulus*). Tables VI, VII and VIII show that the velocity of cataphoresis does not depend to any measurable extent on the bulk conductivity of the particle.

CONCLUSION.

The electrophoretic mobility of microscopically visible particles is independent of size, shape and conductivity of the particle within the limits of the experimental error. This is valid for extreme variations in size, shape and conductivity.

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ANNOUNCEMENT

Volume VIII of *The Journal of General Physiology* is a memorial to its founder, Dr. Jacques Loeb. This volume contains papers by Dr. Loeb's pupils and his associates, together with a portrait and a sketch of his life, and appeared simultaneously with Volumes IX, X, and XI.

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THE RELATION BETWEEN TEMPERATURE AND THE PEDAL RHYTHM OF *BALANUS*.

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(Accepted for publication, January 28, 1929.)

The characteristic contractions and expansions of the pedal cirri,—a respiratory and feeding mechanism,—in the rock barnacle, *Balanus balanoides* L., have proved to be reliable indicators of stimulation. The animal as a whole, and the cirri alone, are very sensitive to chemical, mechanical, photic, and thermal stimuli, the effects of which may be measured by observing the corresponding changes in the character and rate of the pedal rhythm. It is possible therefore to investigate such stimuli qualitatively and in some cases quantitatively, either separately or in appropriate combination, while the others are so far as possible kept constant. A preliminary study of this sort was recently made¹ on one group of *B. balanoides*. The relations between temperature and changes in the pedal rhythm are described in this paper.

Consistent results from changes of temperature are obtained only when the other external conditions are kept as constant as possible. The effects of various external factors, as well as of internal conditions, if not controlled, would lead to an erroneous conclusion concerning the behavior of the barnacle. Here is an animal activity influenced by several factors, but it would be absurd to conclude that their effects cannot be studied separately. If the various factors can be determined and controlled the analysis of any one becomes possible. Merely because an activity is "complex" is not sufficient reason for abandoning efforts to analyze it (*cf.* Herber and Slifer, 1928). In the laboratory regular movements of the cirri demand running water, with a rate of flow within definite limits. This was obtained by means

¹ The experiments were done during the summer of 1928 at the Mount Desert Island Biological Laboratory, where Director Dr. H. V. Neal extended many appreciated courtesies.

of a water gauge which registered the flow in cubic centimeters per minute within an error of 2 per cent. To prevent as much as possible contact stimulation by particles in the water without decreasing the food supply, the water was filtered through cloth with a 1 mm. mesh. Contact stimuli due to feces were rare since the latter sank upon extrusion. Vibrational stimuli were decreased to a minimum by mounting the aquarium on rubber balls set on a layer of moderately soft sheet rubber 15 mm. thick. Illumination was furnished by northern sky light or by Mazda lamps the intensity of which did not change enough during an experiment to elicit the well known shading reflex. To avoid effects due to changes in the food supply or to the physical constants of the water such as pH, specific gravity and salinity, the experiments were done only when any variations in those factors were physiologically insignificant. Interfering stimuli caused by moulting or defecation were eliminated from the results by omitting observations during and immediately following those activities.

A group of 41 animals attached to a stone was placed in a glass aquarium of 650 cc. capacity. The water entered at the bottom at the rate of 250 cc. per minute and overflowed around the top. The resultant immersion of these intertidal animals, continuous for 9 weeks, had no ill effect that could be determined. The temperature of the water was controlled by sending the current through a lead coil (1.5 m. long) immersed in a 12 liter water bath, to which ice, or cold or hot water was added as desired. The temperature of the aquarium water was changed between 0.0° and 28.0°C . either upward or downward by not more than 3° at a time, and not oftener than once in 40 minutes. Allowing 15 minutes for equilibrium the temperature remained constant to within 0.1° for periods of from 15 to 60 minutes, depending upon the ratio of the temperatures of the air and the bath. At each temperature the time required for 10 contractions and expansions was measured by a stop-watch to 0.05 second. The averages of 5 such readings were found to be significant because of the remarkable regularity of the movement under constant conditions. Complete records of 17 individuals, the basal diameters of which varied from 4 to 15 mm., were secured over a period of 9 weeks, involving 965 averages of 6,775 observations at 186 different temperatures between 2.0° and 27.0°C . Twelve hundred observations on 10 other animals

showed similar results but are not included in this report because of their incompleteness over an adequate range of temperatures.

In general, the rate of the pedal rhythm of the barnacle increased regularly with temperature from 2.0° to 21.0°C. , revealing a critical temperature above which the increment was slightly decreased. Below 2.0° the movements were extremely irregular and often spasmodic. Between 21.0° and 27.0° three different effects appeared in different cases: (1) the rate decreased or became irregular, (2) the rate continued to increase, or (3) the valves closed. Beyond 27.0° closure of the valves regularly occurred. In the case of the few animals whose rates continued to increase beyond 21.0° closure at 27° was sudden and not preceded by irregularity. Alteration of the rate by temperature was due primarily to a change in the duration of the expanded phase, without much change in the contraction period, a fact easily demonstrated at the lower temperatures. It may be said therefore that temperature effects in the barnacle are primarily "diastolic."

In any one animal the frequency of movement at a given temperature on different days varied only about 5 per cent. Since this latitude of variation was constant throughout the temperature range, data obtained over a long period were combined. Having once determined the relation between frequency and temperature it was possible to predict the temperature of the water to within 1.0° from observations on the rate of movement. Although different animals exhibited different absolute values, two intersecting "hyperbolas" were always obtained when time was plotted against temperature. It would be expected therefore that if the logarithm of the rate were plotted against $1/T^{\circ}$ the points would fall on two intersecting straight lines, indicating the applicability of the so called Arrhenius equation for temperature effects. Separate plots for the 17 animals were so made, and in every case the points could best be represented by two intersecting straight lines. The values of μ (obtained from the slopes of the lines) fell easily into three classes, so that the plots in each class could be combined, it being necessary in some cases to multiply the ordinates by a constant to bring the lines nearer coincidence. These plots, from which a few duplicate points have been omitted for clarity, are reproduced in Figs. 1-3, and show how closely the Arrhenius equation fits the data.

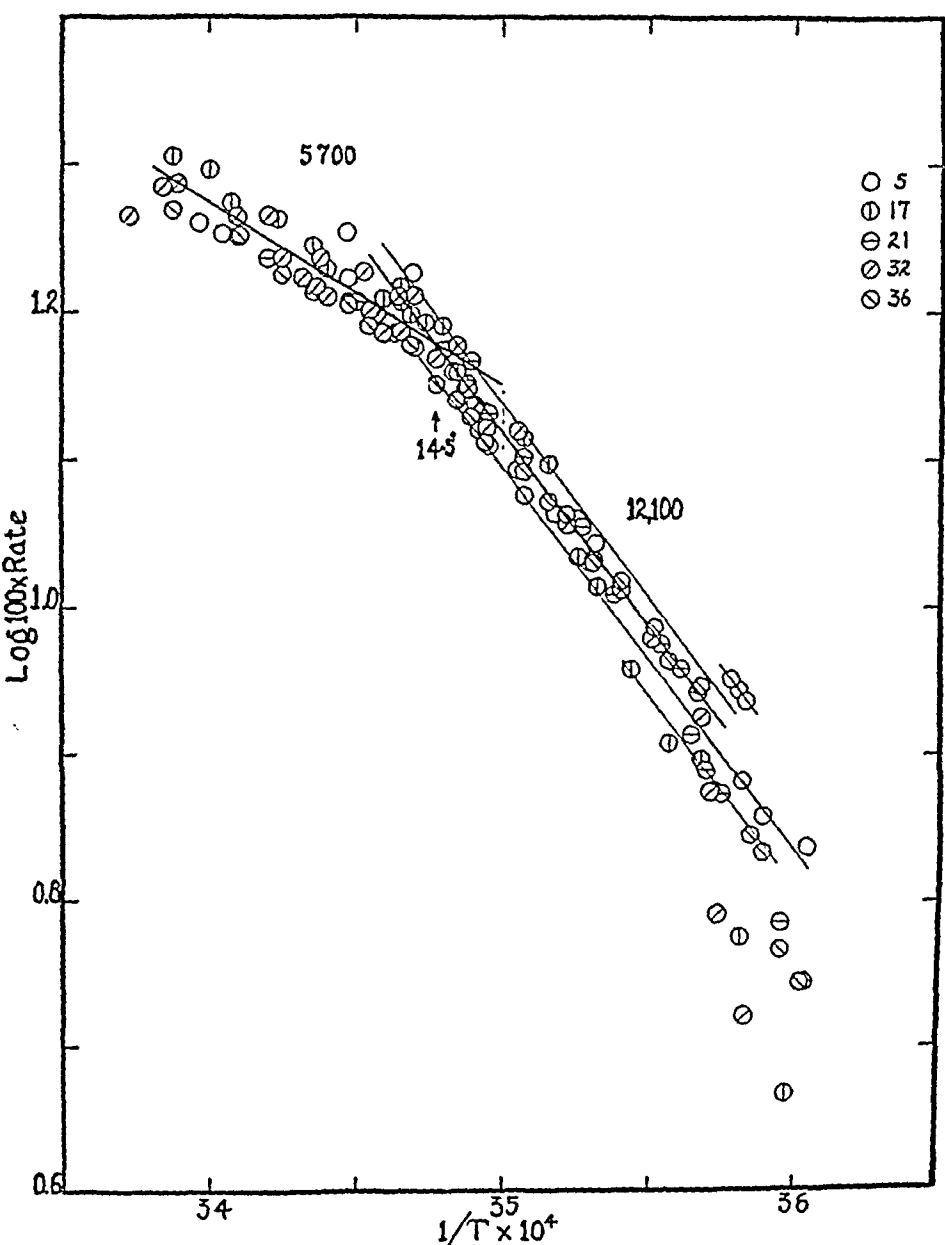


FIG. 1. Data on the rate of the pedal rhythm of *Balanus*, from 5 animals, between 4.4° and 23.4°. Critical temperature is at 14.5° above which $\mu = 5,700$, and below which $\mu = 12,100$. Irregularities appear below 7.0°.

In Fig. 1 are the data from 5 animals, which yielded temperature characteristics of 5,700 from 14.5° to 22.5° , and 12,100 from 7.0° to

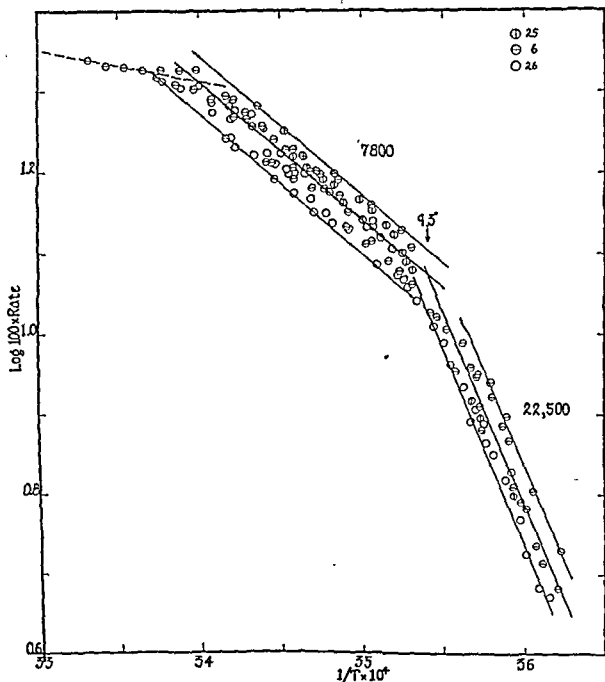


FIG. 2. Data on the rate of the pedal rhythm of *Balanus*, from 3 animals between 2.4° and 27.1° . Critical temperature is at 9.3° above which $\mu = 7,800$ and below which $\mu = 22,500$.

14.5° . Below 7.0° the results were very irregular. In Fig. 2 the values were 7,800 from 9.3° to 21.0° , and 22,500 from 3.1° to 9.3° . Animal 6 in this group continued to show an increasing rate, with a

smaller increment, up to 27.0° , at which closure was sudden. In Fig. 3 the data from 9 animals showed temperature characteristics of

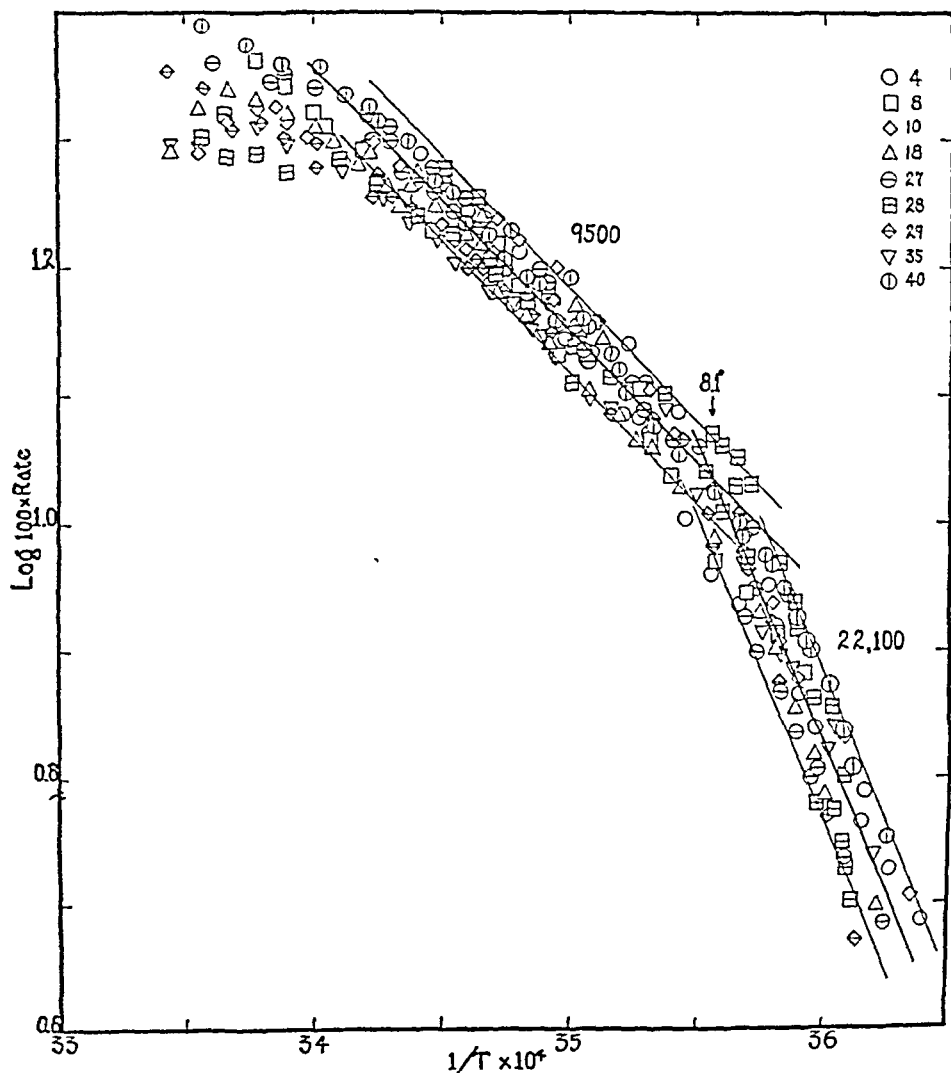


FIG. 3. Data on the rate of the pedal rhythm of *Balanus*, from 9 animals between 1.6° and 25.8° . Critical temperature is at 8.1° above which (to 21.0°) $\mu = 9,500$, and below which $\mu = 22,100$.

9,500 from 8.1° to 21.0° and 22,100 from 2.4° to 8.1° . Above 21.0° the plot showed several rates increasing slowly up to closure. Different temperature characteristics from different animals over the same

temperature range may be due to the fact that different members of a catenary series of reactions which determines the pedal rhythm assume control of the series under differing conditions of age, sex, nutrition, and the like.

The values of μ obtained for the barnacle are similar to those reported for various other animal activities. It is not surprising to find values near 8,000 and 22,000 for a mechanism which is at least partly respiratory, the former frequently associated with processes involving gaseous exchange. The characteristic of 12,100, however, has been associated with non-respiratory neuromuscular rhythms in arthropods (*cf.* Crozier, 1924; Crozier and Stier, 1925, *a*; 1925, *b*). Wolf (1928) found the same combination of 8,000 and 22,500 in normal *Gonionemus* for frequency of pulsation, which is also a respiratory and food-getting function. These three increments might be expected for an activity which is concerned partly with respiration but operated by a neuromuscular mechanism, if the general indications of the results from temperature analyses are sound.

Increments of about 9,000 calories have been previously found for several different organic processes, such as movement of *Oscillatoria* (Crozier and Federighi, 1924; Navez, 1928), heart beat in *Tiedemannia* (Glaser, 1925), and in *Notonecta* (Crozier and Stier, 1927), and for growth of sporangiophores (Castle, 1928). In some of these cases, however, the value appeared only infrequently and was not considered typical for the process. That this 9,500 is not due to a mixture of values near 8,000 and 11,000 is shown by the following series of values calculated from the individual plots: 8,900, 9,030, 9,040, 9,470, 9,560, 9,600, 10,200, 10,370, 10,400, the average of which is 9,620. So far it has been impossible to detect its significance. The records of $\mu = 5,700$ from the barnacle and $\mu = 4,800$ for locomotion of *Planaria* (Cole, 1926) stand by themselves and any attempt to correlate them with known chemical or biological processes must await further investigation; particularly since they occur at such relatively high temperatures. The critical temperatures revealed in the three sets of data are very close to those reported in many other activities. Changes in the control of the rate of a series of reactions frequently occur near 9.5° and 15.0°; near 8.0° such changes are not so common (*cf.* Crozier, 1926).

Fig. 4 is a mass plot of the results from all the animals and illustrates very clearly the inability to interpret temperature effects

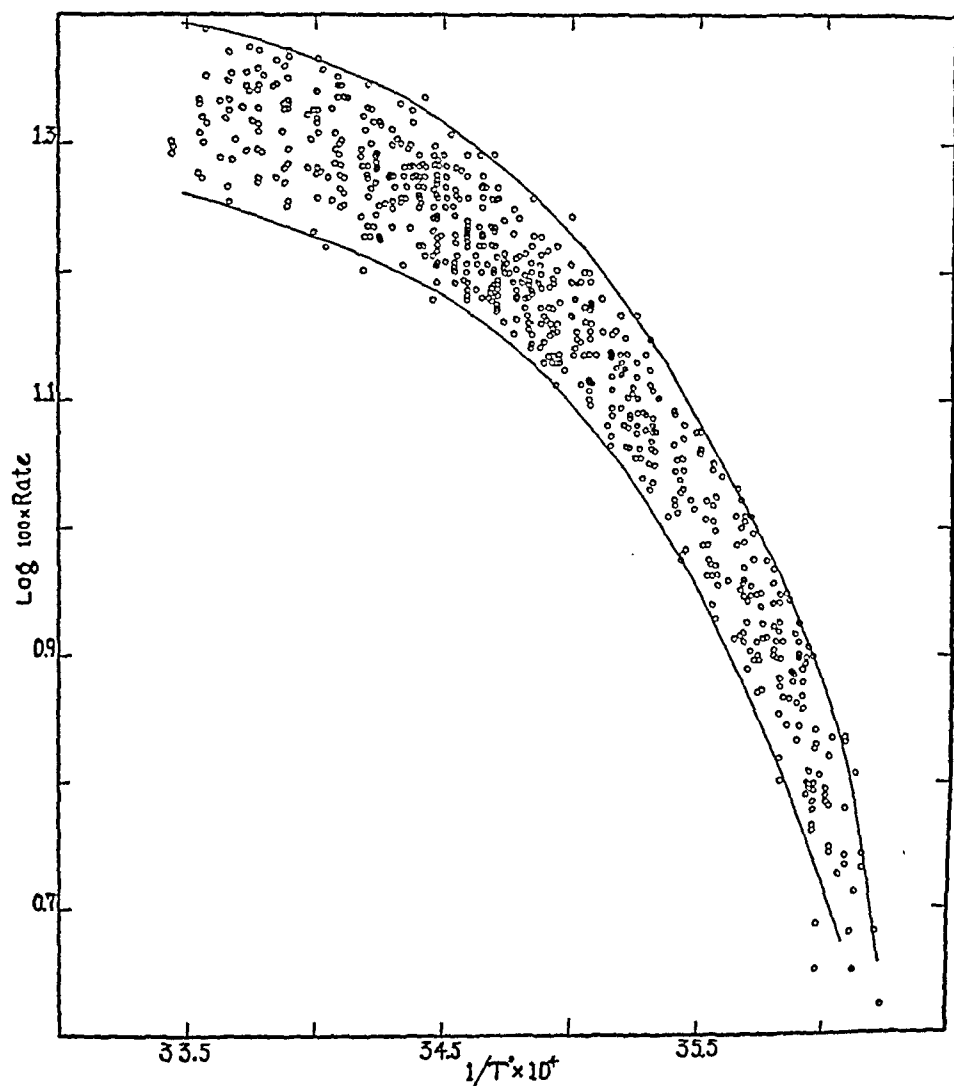


FIG. 4. Data on the rate of the pedal rhythm of *Balanus* from 16 animals between 2.9° and 26.0°, plotted *en masse*. Curvilinear lines indicate different temperature characteristics among the different individuals and the necessity of individual plots.

correctly when the animals show individual differences. The curvilinear shape of the best fitted line is due to the different temperature characteristics over the medium range of temperatures, and to the

varying irregularities above 21.0°C. (Crozier, 1924; Crozier and Stier, 1925, *a*; Glaser, 1924.) If the Arrhenius equation is to be applied to such data individual plots must be made (*cf.* Castle, 1928, page 412; Glaser, 1925).

A second series of experiments was performed to detect any effect of diluting the sea water upon the temperature relations, the pH being kept constant. Enough observations were collected to demonstrate that any dilution greater than 4 per cent caused irregularity of movement; that 3 and 4 per cent dilutions progressively decreased the rates, without altering the temperature characteristics; and that dilutions of 2 per cent or less caused no significant change in rate. The results indicate the extreme sensitivity of this barnacle to fresh water as far as regularity of movement is concerned. It is well known however that much larger amounts of fresh water are tolerated by barnacles without permanent injury, although the pedal rhythm may be temporarily affected.

SUMMARY.

1. The relation of temperature to the pedal rhythm of *Balanus balanoides* L. has been studied under otherwise constant conditions.

2. The frequency of movement increases with temperature, showing three groups of thermal increments and three critical temperatures. Five animals yielded $\mu = 5,700$ above 14.5° C. and 12,100 below; 3 gave $\mu = 7,800$ above 9.3° and 22,500 below; while 9 showed $\mu = 9,500$ above 8.1° and 22,100 below.

3. The upper critical temperatures, above which different effects appeared in different animals were 23.4°, 26.0°, and 27.0°. Above 27.0° none of the valves remained open.

4. Excepting the values 5,700 and 9,500, the increments are similar to those previously found to be associated with respiratory and with neuromuscular activities.

5. Dilution of the sea water with from 3 to 4 per cent fresh water decreases the rate without altering the increments. More than 4 per cent dilution causes irregularity.

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THE RELATION BETWEEN MAXIMUM RATE OF PHOTOSYNTHESIS AND CONCENTRATION OF CHLOROPHYLL.

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(Accepted for publication, January 28, 1929.)

I.

Most investigations of photosynthesis have depended on measurement of the effects of varying certain external conditions on the rate of this process. It is recognized that "internal factors" also have an effect on the rate of photosynthesis. Internal factors are mentioned by both Blackman (1905, p. 281) and Spoehr (1926, p. 161), but owing to the difficulty of controlling them few have tried to investigate the part they may play. Chlorophyll, the most tangible internal factor, has attracted the attention of numerous investigators. Some have tried to study the relation of different concentrations of chlorophyll to the rate of photosynthesis. But no one prior to Willstätter and Stoll accomplished much in this field, chiefly because the nature of the leaf pigments was not understood, and their quantitative determination was impossible.

Willstätter and Stoll, having devised satisfactory methods for the quantitative determination of leaf pigments (1913), undertook to study photosynthesis with leaves containing different concentrations of chlorophyll. They established beyond doubt the fact that during photosynthesis the amount of chlorophyll remains unchanged, even during prolonged exposure to strong light at high temperatures. Pfeffer and Czapek, among others, had suggested that during photosynthesis chlorophyll may be decomposed and reformed constantly. Willstätter and Stoll show that no real decomposition of chlorophyll takes place during photosynthesis. I quote from them (1918, p. 11) the following table:

* National Research Council Fellow.

*Chlorophyll in 10 g Blättern vor und nach der Assimilation.*5 proz. CO₂, ungefähr 75000 Lux.

Pflanze	Temperatur und Assimilationszeit	Chlorophyll (mg.)	
		vor der Assimilation	nach der Assimilation
Prunus Laurocerasus.....	30°, 6 Stunden	12,2	12,4
“ “	30°, 22 “	9,4	9,5
Hydrangea opulodes.....	30°, 6 “	9,2	9,1
Pelargonium zonale.....	40°, 6 “	12,5	12,8

They show in the same chapter (see p. 32) that not only is the total amount of chlorophyll unaffected by rapid assimilation, but neither is the ratio between chlorophyll *a* and *b*.

Nevertheless, they were unable to establish any relationship between chlorophyll content and capacity for photosynthesis. They used leaves from green and yellow varieties of the same species, yellowing autumn leaves, greening spring leaves, etiolated, and chlorotic leaves. Their results are expressed as amounts of CO₂ reduced per unit amount of chlorophyll. The following figures, quoted from Willstätter and Stoll (1918, p. 43), show some of the widely differing values they obtained:

One molecule of chlorophyll assimilates per hour:

In old autumn leaves of *Ampelopsis quinquefolia* 18 molecules of CO₂.

In young autumn leaves of *Ampelopsis quinquefolia* 164 molecules of CO₂.

In summer leaves of *Sambucus nigra* 135 molecules of CO₂.

In summer leaves of *Sambucus nigra* var. *aurea* 2463 molecules of CO₂.

In etiolated leaves of *Phaseolus vulgaris* 2736 molecules of CO₂.

The experiments of Willstätter and Stoll were carried out under the usual difficulties involved in working with leaves. The temperature of the cells of an assimilating leaf is never accurately known. The palisade cells of dark green leaves screen the internal and lower cells so that all are not equally illuminated. In yellow leaves, on the other hand, the light is much less weakened in traversing the cells, so they are more equally illuminated.

These may be some of the reasons why the results of Willstätter and Stoll fail to reveal any correlation between amount of chlorophyll and rate of photosynthesis. But in spite of Spoehr's statement

(1926, p. 153) that Willstätter and Stoll have definitely established the disproportionality between chlorophyll content and assimilation, I think we must agree that if chlorophyll really takes part in photosynthesis its concentration should bear some regular relation to the rate of the reaction. The results of Willstätter and Stoll show that among a wide variety of leaves chlorophyll shows great variation in activity. They do not show, however, that such variation exists in leaves chosen to be comparable in all respects other than in chlorophyll content.

The experiments described in this paper were undertaken to see if by using comparable cells, differing so far as possible in chlorophyll content only, some regular relationship between chlorophyll and photosynthesis could be established. Such a relationship, if established, would afford experimental proof that the green pigment chlorophyll is really concerned in photosynthesis.

The advantages of working with a unicellular alga in aqueous suspension have been emphasized by Warburg. For studying photosynthesis at high light intensities suspensions containing very few cells may be used, so that all cells are about equally illuminated. In thin suspensions the temperature of the cells cannot differ significantly from that of the surrounding fluid, which may be kept very constant. On account of its various conveniences, therefore, the green alga *Chlorella vulgaris* was used for this investigation.

II.

Method of Culturing Cells.

Spoehr states (1926, p. 142) that chlorophyll content cannot be artificially varied. However, the chlorophyll content per unit volume of cells of *Chlorella vulgaris* has shown itself to be remarkably sensitive, especially to the composition of the culture medium.

The method of culturing was essentially that described by Warburg (1922, p. 427) in his papers on photosynthesis. As this is already adequately described, I shall add here only such modifications as were used for controlling the chlorophyll concentration per unit volume of cells.

The cultures were grown in 300 cc. Erlenmeyer flasks with tubes

sealed into the tops for passage of a stream of gas. The entrance and exit tubes were plugged with cotton, and the flasks, each containing 225 cc. of medium, were sterilized and inoculated with 3 cc. of cell suspension by means of sterile pipettes. Usually from 10 to 30 c. mm. of cells were inoculated into each flask, each flask in a given set receiving exactly the same amount.

The medium was prepared as Warburg recommends (1922) but only one half the amounts of his stock solutions were used per liter of medium. To dilute the stock solutions, a solution of 1.5 per cent glucose was prepared, and an excess of calcium carbonate added, the suspension well mixed, autoclaved 20 minutes at 13 pounds pressure, mixed again, and allowed to cool and settle slowly. The supernatant glucose solution was decanted and used for diluting the stock solutions. This procedure of autoclaving with calcium carbonate is recommended by Steinberg (1919, p. 358) for removal of traces of heavy metals from nutrient media. It was found that without this treatment chlorophyll concentration could not be varied over so wide a range, probably because of the relatively large amount of iron present in the glucose. Merck's glucose C.P. was used, and best grade Kahlbaum salts; water redistilled from a Pyrex still was used exclusively. The prepared medium was of the following composition:

MgSO ₄	0.01 molar
KNO ₃	0.0125 "
KH ₂ PO ₄	0.0090 "
CaCO ₃	0.0001 "
Glucose.....	about 1.5 per cent

A number of experiments were made, a summary of two of which appears in Table I, to determine whether glucose in this concentration depresses photosynthesis. Numerous writers (see Spoehr, 1926, p. 159) have stated that the accumulation of end-products materially cuts down the rate of photosynthesis. It might be expected that a 1.5 per cent glucose solution would show a marked inhibitory effect. No such effect could be demonstrated, the rate of photosynthesis being the same with and without glucose. The respiration of cells in glucose solution was higher, showing that the glucose had penetrated the cells.

The use of glucose in the culture medium of an autotrophic organism

like *Chlorella* is of course unnecessary for growth. But the method used for controlling chlorophyll content was to vary the iron concentration, and glucose was needed to facilitate growth at low concentrations of iron. With only traces of iron present as impurities in the salts and glucose, *Chlorella* soon becomes heterotrophic, loses all its chlorophyll, and continues to grow and live by using glucose. The writer has used this technique of culturing *Chlorella* in glucose solution before (1927). It readily returns to the autotrophic condition and develops chlorophyll if it is returned to a medium with plenty of iron, with or with-

TABLE I.

No. of cells	Suspension fluid	Glucose concentration	Time	O ₂ evolved
mm. ³		per cent	min.	mm. ³
32	Knop*	0	10	43.8
32	Knop	4	10	49.0
10	Carbonate mixture	0	15	27.0
10	Carbonate mixture	1.5	15	27.2

* When Knop solution was used, it was saturated with 5 per cent CO₂ in air.

TABLE II.

Cc. Fe ₂ (SO ₄) ₃ stock added to culture	Mm. ³ cells used to determine Δh	KO ₂	Δh	CO ₂	Mm. ³ cells extracted	ϕ_1	ϕ_2	* per 10 mm. cells
			per hr.					
0.25	15.5	0.54	+332	+126	36.5	57.5°	34.0°	0.101
0.025	8	0.56	+155	+118	38	57.5°	33.4°	0.099

out glucose. In glucose solutions poor in iron its growth is of course slower than with plenty of iron, but the rate of growth is more nearly the same than if no glucose is used. If one tries to control chlorophyll concentration by growing cultures without glucose and with graded iron concentrations, the cultures poorest in iron grow very slowly, turn yellow and sickly, and show abnormal metabolism, so that they are in no way comparable with cells grown on media richer in iron. For the same reason the leaves of plants grown in water cultures with different amounts of iron are not comparable. Cells deprived of iron, and without sufficient chlorophyll, cannot

remain normal unless they are supplied with carbohydrate from the outside. Glucose was used in these cultures to obtain more nearly equal growth with different amounts of iron. Iron was added from a stock solution containing 2.1 gm. of ferric sulfate per liter. Amounts varying from 0.50 to 0.01 cc. of this stock solution were added to each culture of 225 cc. With this range of added iron, and using glucose, pure cultures of *Chlorella* may be grown at roughly comparable rates, producing cells of about equal size and equal rates of respiration, but differing widely in chlorophyll content.

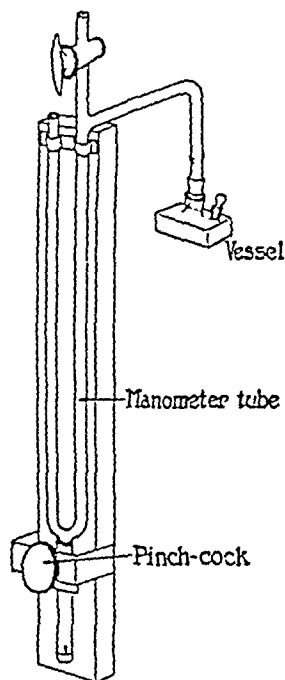


FIG. 1.

The rate of photosynthesis is independent of the iron concentration in the culture, up to the point where iron concentration begins to affect chlorophyll concentration. Up to the age of 3 to 4 days, all cultures produce cells of nearly the same chlorophyll content, no matter what the iron concentration. Table II shows rate of photosynthesis and the chlorophyll content for two cultures, one containing ten times as much iron as the other. Rate of photosynthesis per unit volume of cells and chlorophyll content per unit volume of cells are nearly the same for each culture. After 4 or 5 days, the cultures poorer in iron begin to show lower chlorophyll concentration, and with this the rate of photosynthesis falls off. But external iron concentration alone apparently does not

affect rate of photosynthesis.

Cultures were grown in a glass-bottomed water-bath kept at 19–21°C., and illuminated from below with three 40 watt internally frosted concentrated filament lamps about 30 cm. from the bottoms of the culture flasks. A continuous stream of air containing 5 per cent CO_2 was passed through the cultures.

III.

Methods of Measuring Photosynthesis and Chlorophyll Concentration.

Photosynthesis was measured manometrically, by a method well worked out and described by Warburg (1924). Fig. 1 shows the

type of vessel and manometer used. The total volume of each vessel, to the level of the manometric fluid, was about 12 cc. 7 cc. of cell suspension were pipetted into each vessel, so that the total gas space was about 5 cc. Under these conditions, 1 c. mm. of oxygen evolved at 20°C. causes a change of pressure equal to about 2 mm. of Brodie's solution (the fluid used in the manometer). To find the amount of oxygen evolved in photosynthesis, the change in pressure was multi-

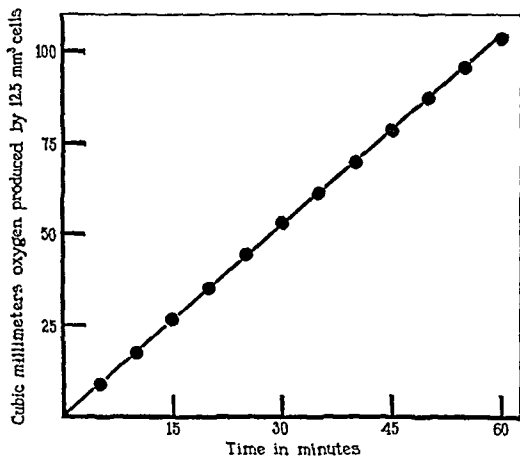


FIG. 2.

plied by a constant, K_{O_2} , characteristic for each vessel for a given temperature and volume of suspension. For the mode of calculation of K_{O_2} , consult Warburg (1924).

The cells were centrifuged out of their culture medium, washed in a mixture of 15 cc. M/10 Na_2CO_3 + 85 cc. M/10 $NaHCO_3$ (Warburg's "Carbonate Mixture No. 9"), and suspended in this mixture for the measurement of photosynthesis. The concentration of CO_2 in this mixture is so high that the rate of photosynthesis is independent of

small changes of CO_2 concentration. The mixture has, according to Warburg (1919, p. 238) an OH^- ion concentration of $10^{-4.8}$, and contains 91×10^{-6} moles of CO_2 per liter. Variation with temperature is comparatively small, the CO_2 concentration dropping probably to about 71×10^{-6} moles per liter at 5°C . Warburg states that for the organism he used, *Chlorella pyrenoidosa*, this mixture was not injurious for many hours. I found that *Chlorella vulgaris*, although it would continue to respire and to assimilate CO_2 in this mixture for a day, showed a gradual decrease in rate of photosynthesis after the 1st hour and a half. This decrease amounted to about 20 per cent after 5 hours. Each measurement was carried out without keeping the cells in the carbonate mixture longer than 45 minutes. During this time the rate remained nearly constant. Fig. 2 shows amount of photosynthesis plotted against time for the 1st hour for a typical set of cells.

The carbonate mixture is buffered for CO_2 . When 1 molecule of CO_2 is withdrawn for photosynthesis, 2 molecules of bicarbonate decompose to form carbonate and carbonic acid:



The only changes in pressure are therefore due to the oxygen used in respiration and that evolved in photosynthesis. The ratio $\frac{\text{CO}_2}{\text{O}_2}$ need not be considered if the measurements are taken in the carbonate mixture. Oxygen production is a better measure of photosynthesis than carbon dioxide consumption because readings of the latter might be influenced by fermentation of the glucose present in the cells.

Warburg has calculated the "resistance" of the buffer action (capacity to furnish CO_2 at constant pressure) of this carbonate mixture, and finds that in 10 cc. of carbonate mixture 0.2 cc. of CO_2 may be used up without altering the CO_2 tension more than 10 per cent. The evolved oxygen resulting from the reduction of 0.2 cc. of CO_2 would cause under my conditions a change of pressure of about 400 mm. of Brodie's solution. In no single experiment did the total amount of photosynthesis ever approach this value. Variations in CO_2 concentration were consequently well under 10 per cent.

The most satisfactory source of light for high intensities was found

to be a tungsten ribbon lamp using 6 volts drawn from the 110 volt alternating current lighting circuit by a transformer. The lamp was mounted outside the thermostat, its light made nearly parallel by means of a lens, and thrown on the cell suspension through a plate glass window in the side of the thermostat. In this way intensities of over 100,000 lux were obtained. This arrangement proved to be superior to that involving bulbs immersed in the thermostat, because it made possible the use of intense light without overheating the thermostat water. At times when very intense light was not desired, ordinary concentrated filament lamps were placed directly in the thermostat, their sockets being imbedded in paraffin blocks.

Chlorophyll concentration per unit volume of cells was determined by washing a known volume of cells with distilled water, and extracting with methyl alcohol until all soluble pigment was extracted. The total extract was made up to 25 cc. in a volumetric flask. The extinction coefficient ϵ of a sample of this solution, containing chlorophyll, carotin, and xanthophyll, was determined for the wave-length $\lambda = 670 \mu\mu$. This point lies completely outside the absorption range of carotin and xanthophyll, and is in the region of maximum absorption by chlorophyll in the red. ϵ was measured on a König-Martens spectrophotometer, the two angles ϕ_1 and ϕ_2 being read at equality of the halves of the field. The glass block used in the absorption vessel was 10 mm. thick. Therefore

$$\epsilon = \log \tan \phi_1 - \log \tan \phi_2$$

If x is the number of cubic millimeters of cells extracted $\frac{\epsilon_{670}}{x} \times 10$ is the extinction coefficient of the chlorophyll of 10 c. mm. of cells made up to 25 cc. in methyl alcohol. This value, ϵ per 10 c. mm. of cells, is proportional to the concentration of chlorophyll alone, because the yellow pigments do not absorb in this part of the spectrum. The values of ϵ given in the following pages are of course relative, but they may be translated into absolute units at any time by measuring ϵ for an alcoholic solution of chlorophyll of known concentration. No attempt was made to determine chlorophyll (*a*) and (*b*) separately. Willstätter has shown that their ratio is relatively constant wherever chlorophyll occurs, and that it remains unchanged during photosynthesis.

IV.

The Relation of Chlorophyll Concentration to Rate of Photosynthesis.

Sets of from 3 to 5 cultures were grown for periods of from 5 to 7 days; to each culture of 225 cc. was added an amount of ferric sulfate varying from 1.05 mg. down to zero. Each culture of a given set was started with the same amount of inoculum. When the cultures had developed enough to show a good range of chlorophyll concentrations, all were removed from the illuminated bath where they were grown, and placed in the dark, in the refrigerator, a procedure which did not impair their photosynthetic capacities. The rate of photosynthesis at high light intensity was determined for a sample of

TABLE III.

Culture No.	Cc. Fe stock solution added to culture	Rate of respiration mm. ³ O ₂ used for hr. per 10 mm. ³ cells	ϵ_{470}
1	0.0	11.0	0.036
2	0.025	6.7	0.045
3	0.050	8.7	0.063
4	0.100	7.7	0.080
5	0.250	7.8	0.085

cells from each culture, three readings being made with each sample. At the same time another sample of cells from each culture was extracted with methyl alcohol for determining chlorophyll concentration. Usually 5 to 20 c. mm. of cells in 7 cc. of carbonate mixture were used for each measurement of photosynthesis. With such thin cell suspensions, the amount of photosynthesis is directly proportional to the quantity of cells, showing that the light is not appreciably weakened by passing through the cell suspension. Larger quantities of cells were used when the chlorophyll concentrations were very low. However, this was permissible because the blue light, which is much more absorbed by the thick suspensions on account of their carotin and xanthophyll content, was present in the light source in such small amounts relative to the light which was unabsorbed by the yellow pigments, that it was of no importance in the rate of photosynthesis. This was established by measuring photosynthesis with the light beam

passing through an orange glass filter cutting out all the light absorbed by the yellow pigments. Removal of the orange glass caused no change in the rate of photosynthesis.

Details of the measurement of photosynthesis were as follows: A measured amount of cell suspension, containing a known volume

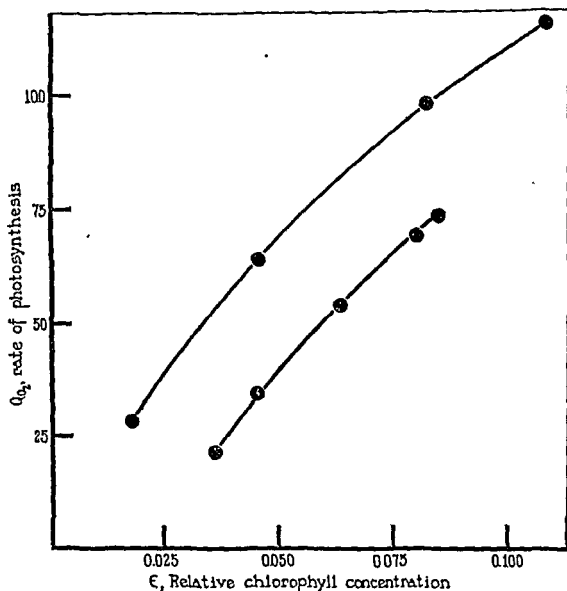


FIG. 3. .

of cells, was centrifugally washed with the carbonate mixture, and made up to a definite volume with the carbonate mixture. 7 cc. of this suspension were pipetted into a vessel, the latter was connected with its manometer, and illuminated in the thermostat for a preliminary period of 15 minutes. Readings were taken at intervals of 5

or 10 minutes. It was found that respiration did not vary with chlorophyll content, so a uniform correction for respiration was made. Table III shows rates of respiration for four different concentrations of chlorophyll. It must be remembered that these values for "rate of respiration" were obtained from thin cell suspensions, made up for determining photosynthesis. The latter is a much more rapid process. Conditions were therefore not favorable for accurate measurement of respiration. The variations seen in Table III are not beyond the error of the measurements, and there is no tendency for respiration to vary uniformly with chlorophyll concentration. The rate of photosynthesis, Q_{O_2} , expresses the number of cubic millimeters of oxygen produced by 10 c. mm. of cells in 1 hour. If

Δh = number of mm. change of manometer pressure per hour

r = number of mm.³ O₂ used in respiration per hour

K_{O_2} = constant of the vessel for oxygen

n = number of c. mm. of cells used for determining Δh

then

$$Q_{O_2} = \frac{\Delta h \times K_{O_2}}{n} \times 10 + r$$

For the determination of ϵ a larger volume of cells was used than for the determination of Q_{O_2} . ϵ was calculated as described in section III.

Fig. 3 shows values of Q_{O_2} plotted as ordinates against ϵ as abscissæ, for two different sets of experiments. Detailed data for the upper curve, covering the widest range of chlorophyll concentrations studied, will be found in Table V. The coordinates of the points on both curves are given in Table IV.

Although the curves are not superimposed, both show clearly that the rate of photosynthesis at high light intensities is a smooth function of chlorophyll concentration. The relation between the two is nearly linear. It is reasonable to suppose that if all conditions for both sets of cultures had been identical, the curves would be superimposed. However, it would be difficult to maintain such factors as the age, and the amount and character of the inoculum constant from one set of culture vessels to another. Furthermore, it is a matter of minor importance whether the curves of different sets are superimposed. The important point is that in every set studied, the points lie on a smooth curve similar in character to the two depicted in Fig. 2.

It is easy to see how such divergent values as were published by Willstätter and Stoll could be obtained by comparing points on one curve of Fig. 3 with points on the other curve. Take for example the two lowest points. The lowest point on the shorter curve represents about twice the chlorophyll content of the lowest point on the

TABLE IV.

	Cc. $\text{Fe}(\text{SO}_4)_2$ solution added to culture	Q_{O_2}	ϵ_{470}
Curve A	0.000	28.4	0.018
	0.010	64.0	0.045
	0.025	91.0	0.082
	0.250	115.0	0.107
Curve B	0.000	21.6	0.036
	0.025	34.4	0.045
	0.050	54.0	0.063
	0.100	69.0	0.080
	0.250	73.4	0.085

TABLE V.

Culture	Cc. Fe solution added	Mm. ³ cells used for rate deter- mination	Mm. ³ cells ex- tracted	Δh mm per 10 min.	K_{O_2}	r	Q_{O_2}	ϕ_1	ϕ_2	ϵ_{470} per 10 mm. ³ cells
1	0.00	7	14	+ 4.5	0.53	8	28.4	43.8°	42.1°	0.013
2	0.010	12.5	19.0	+21.5	0.54	8	64.0	46.9°	41.3°	0.045
3	0.025	9.5	19.0	+23.5	0.56	8	91.0	48.7°	38.5°	0.082
4	0.25	6.6	26.4	+19.6	0.60	8	115.0	52.3°	34.1°	0.107

$$\epsilon = \frac{\log \tan \phi_1 - \log \tan \phi_2}{\text{mm.}^3 \text{ cells extracted}} \times 10$$

$$Q_{O_2} = \frac{K_{O_2} \times \Delta h \text{ per hour}}{\text{mm.}^3 \text{ cells}} \times 10 + r$$

longer curve, yet the rate of photosynthesis is slightly lower for the higher chlorophyll content. Equal amounts of chlorophyll do not cause equal rates of photosynthesis when one set of cultures is compared with another. The curves of Fig. 3 present, I believe, the first direct piece of experimental evidence that chlorophyll is the pigment concerned in photosynthesis. Clearly the rate of this process, besides depending on various external factors such as temperature, illumination, and carbon dioxide, is also a function of the amount of chloro-

phyll present per unit volume of cells. But to bring out the regularity of this function, it is essential that material of different chlorophyll content be strictly comparable in other respects.

The quantity ϵ , referred to as *chlorophyll concentration*, does not necessarily indicate concentration in the chemical sense, but only the amount of chlorophyll present per unit volume of cells. Nothing is known about the concentration of the pigment inside the individual cells. Nevertheless, it may be pointed out that the shape of the curves of Fig. 3 is similar to the shape of curves of rate of photosynthesis as a function of the true concentration of external factors (carbon dioxide and light intensity).

It seems essential to consider chlorophyll in relation to these other factors in any attempt to explain the mechanism of photosynthesis. A paper covering experiments on photosynthesis as a function of temperature and of light intensity at different concentrations of chlorophyll follows the present one.

SUMMARY.

Willstätter and Stoll have studied the rate of photosynthesis as a function of chlorophyll content. However, their experiments fail to reveal any regular relationship between these two quantities, probably because, in order to obtain material differing widely in chlorophyll content, they were obliged to use leaves which were not comparable in other respects. The writer describes a method for varying the chlorophyll content per unit volume of cells of *Chlorella vulgaris*, maintaining other factors constant.

Experiments are described which show that the maximum rate of photosynthesis is a smooth function of the chlorophyll content.

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PHOTOSYNTHESIS AS A FUNCTION OF LIGHT INTENSITY AND OF TEMPERATURE WITH DIFFERENT CON- CENTRATIONS OF CHLOROPHYLL.

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I.

The writer has shown in a previous paper (Emerson, 1929) that the rate of photosynthesis of *Chlorella vulgaris* is a smooth function of the amount of chlorophyll per unit volume of cells. A relationship between rate of photosynthesis and chlorophyll content being established, it seemed advisable to vary chlorophyll in connection with other factors. As Willstätter (1918) points out, this is the only way of finding out the rôle played by chlorophyll in the photosynthetic process. He suggests that it may function in other ways besides its more obvious part in the absorption of light.

Willstätter and Stoll (1918) made measurements of the rate of photosynthesis of leaves containing different amounts of chlorophyll, at different light intensities and temperatures. They concluded that the yellow leaves were more affected by light intensity, the green leaves more by temperature. The writer has pointed out (1929) that their choice of leaves as material for a study of this kind was unfortunate because of the large number of variables introduced. Their results are probably due to other variables besides chlorophyll content, and do not really indicate properties of the photosynthetic process as a function of chlorophyll content. For this reason it was thought desirable to repeat the experiments of Willstätter and Stoll with material more nearly comparable in all respects except chlorophyll content.

The culture methods for producing *Chlorella* cells differing in chlorophyll content, the method of measuring photosynthesis, and the deter-

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mination of chlorophyll per unit volume of cells, are all described in a preceding paper (Emerson, 1929) and need no discussion here.

II.

The Rate of Photosynthesis as a Function of Light Intensity at Different Concentrations of Chlorophyll.

The rate of photosynthesis as a function of the light intensity has been studied by numerous workers (*c.g.*, Pantanelli, 1903; Blackman and Matthaei, 1905; Blackman and Smith, 1911; Warburg, 1919; Harder, 1921). The curve published by Warburg (1925) may be taken as typical. The rate of photosynthesis increases first rapidly with increasing light intensity, then more slowly. At very high light intensities the rate of the process is practically independent of this factor. Willstätter and Stoll (1918) state that to bring the rate of photosynthesis to this maximum, a higher intensity of light is required for yellow leaves than for green leaves. They plot (p. 149) rates of photosynthesis as ordinates against intensity in lux as abscissæ, for leaves of normal and yellow varieties of the elm. Their figure shows that at an intensity of about 24,000 lux the curve for green leaves has bent over parallel to the abscissa, while the curve for yellow leaves is still rising. Their explanation of this is that the chlorophyll in the upper cells of the greener leaves screens the chlorophyll of the lower cells, preventing the latter from reaching their full capacity. The chlorophyll of the upper cells is assumed to be supplied with more light than it can use, even at lower intensities. This would account for a lower efficiency of greener leaves, but could hardly explain the observation that green leaves reach their maximum rate of photosynthesis at a lower intensity of light than yellow leaves. One would rather expect the reverse to be the case, since in yellow leaves there would be very little screening of the lower cells, and *all* the chlorophyll should receive as much light as it could use at a relatively low intensity. Willstätter and Stoll were unable to get an intensity high enough to cause maximum photosynthesis in the yellow leaves. By bringing their lamp very close to the assimilation chamber they achieved intensities of about 75,000 lux, but at the same time they could not avoid overheating the leaves.

The writer used an arrangement already described (1929) whereby intensities of over 100,000 lux were obtainable without overheating the cell suspension. The rays of a tungsten ribbon lamp running at 6 volts and about 17 amperes were collected by means of a lens, made nearly parallel, and directed to the assimilation vessel with mirrors. The vessel was filled with a thin cell suspension (10 to 20 c.mm. of cells in 7 cc. of carbonate-bicarbonate mixture) so that danger of heating through excessive absorption of light was avoided; the temperature was kept constant to within 0.1°C . With such thin suspensions, the rate of reduction of carbon dioxide is directly proportional to the number of cells used, showing that all cells are practically equally illuminated.

The usual method of varying the intensity of the light by changing the distance of the source from the assimilating cells is unsatisfactory at best, because no source can be regarded as a "point source of light," and the nearer it is placed the greater is the deviation from the inverse square law. The method was inapplicable here because parallel light was used. To vary the intensity, non-selective filters of known transmissivity were used. They were sheets of gelatine containing different amounts of carbon, and cemented between parallel sided pieces of colorless glass. For the visible spectrum these filters are non-selective. A set transmitting 5, 10, 25, 50, and 75 per cent of the incident light was used. By using these singly or combined in pairs, the unscreened light intensity of over 100,000 lux was stepped down to 1/400 of this value in seven steps.

The rate of photosynthesis was measured over three successive 5 minute periods at each light intensity. A measurement of respiration, in the dark, followed these three determinations. It was found that the rate of respiration was independent of the intensity of the immediately preceding illumination, so a uniform correction for respiration was made. To avoid the necessity of making a correction for decrease in rate of photosynthesis with time, a fresh set of cells was used at each intensity of light. During an experiment the stock suspension was kept in the refrigerator. Suspensions may be kept in the refrigerator for 36 hours or longer without having their photosynthetic capacity at 20°C . impaired or altered. An adapting time of 15 minutes was found to be sufficient at all light intensities.

From each culture studied, a sample of cells was extracted with methyl alcohol, and the extract made up to 25 cc. The extinction coefficient ϵ_{670} was determined with a König-Martens spectrophotometer. ϵ represents the relative chlorophyll content per 10 c.mm. of cells.

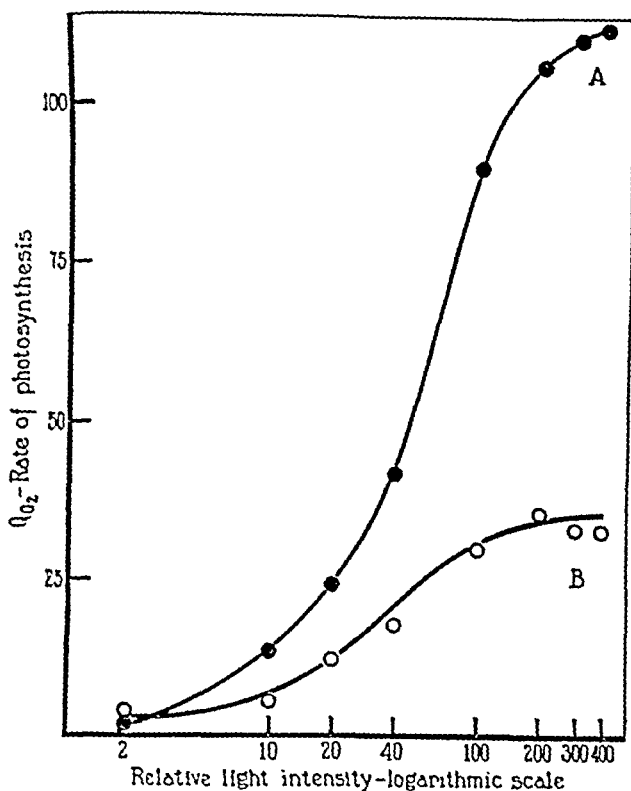


FIG. 1. Curves showing rate of photosynthesis plotted as a function of light intensity, for high and low chlorophyll content per unit volume of cells.

Q_{O_2} , the number of cubic millimeters of oxygen produced per hour by 10 c.mm. of cells, representing the rate of photosynthesis, was plotted against the logarithm of the relative light intensity. The usual method of plotting rate directly against intensity spreads the curve out unnecessarily along the abscissa, and the semilogarithmic plot is more comprehensible.

A large number of experiments were performed, with slight variations in procedure. The results from these individual series led al-

ways to the conclusion that photosynthesis reached its maximum rate at about the same intensity of light, no matter what the chlorophyll content. This is illustrated for two extremes of chlorophyll content in Fig. 1. The value of ϵ for the upper curve is 0.099, for the lower curve 0.026. The coordinates of the points in Fig. 1 are given in Table I, and a summary of the experimental details is given in Table V. The figure shows that both curves bend over toward the abscissa at about the same intensity of light, although they rise to very dif-

TABLE I.
Data for Fig. 1.

	Q_{01}	Per cent transmission of filters used	Relative light intensity	$\log I$
Curve A $\epsilon = 0.099$	112	100	400	2.602
	111	75	300	2.477
	106	50	200	2.301
	90	25	100	2.000
	42	10	40	1.602
	24	5	20	1.301
	13.5	5, 50	10	1.000
	2.1	5, 10	2	0.301
Curve B $\epsilon = 0.026$	32.5	100	400	2.602
	33.1	75	300	2.477
	35.5	50	200	2.301
	33.5	25	100	2.000
	17.8	10	40	1.602
	12.5	5	20	1.301
	5.7	5, 50	10	1.000
	4.3	5, 10	2	0.301

ferent heights. It must be remembered that the scale of the abscissa is logarithmic. The flatness of the tops of these curves would be even more evident in a direct plot. It is interesting to note the shapes of these two curves. They are quite dissimilar, and the upper one cannot be produced by multiplying the bottom one by a constant. The curves start at about the same level, showing that at low light intensities the same amount of photosynthesis may be carried on by much or little chlorophyll. The chlorophyll is more efficient when dilute.

This gives us a key to the concentration of chlorophyll *in the plastid*. If we should vary the amount of chlorophyll in the suspension, instead of chlorophyll content per unit volume of cells, we could make two curves using different amounts of cells alike in chlorophyll content. This would give us two exactly similar curves with ordinates the same distance apart from end to end. Nevertheless, the *amount* of chlorophyll for the lower curve might have been the same as for the lower curve of Fig. 1, only it would be differently distributed in the chloroplasts. The difference in shape of the curves in Fig. 1 is attributable to a difference in the state or condition of the chlorophyll *in the plastid*.

These curves bring out another point which seems to have escaped notice heretofore. At very low light intensities (about 500 lux) the rate of photosynthesis is relatively independent of changes in intensity. At intensities of over 1000 lux, the rate of the process varies rapidly with intensity, and again at very high intensities (around 75,000 lux) the rate becomes comparatively independent of the light intensity. The behavior of the process at intensities above 1000 lux is well known, but it seems that measurements below this value are wanting.

The finding of Willstätter and Stoll that the maximum rate of photosynthesis requires a higher light intensity at lower chlorophyll concentrations must have been due to other differences in their material.

III.

The Rate of Photosynthesis as a Function of Temperature at Different Concentrations of Chlorophyll.

The curves shown by Willstätter and Stoll (1918, p. 155) indicate that leaves poor in chlorophyll are much less sensitive to temperature than greener leaves. Their temperature coefficients for green leaves run above 0.2 higher than for yellow leaves. They say that although both types of leaf are equally well equipped with the "protoplasmic factor," on which it is supposed that temperature acts, the leaf poor in chlorophyll cannot absorb light energy fast enough to keep pace with the "protoplasmic factor" at higher temperatures. This explanation is in accordance with Blackman's view of photosynthesis as a process the rate of which is determined by that factor which is most wanting (F. F. Blackman, 1905). He recognized that the rate

of photosynthesis is affected by temperature at high light intensities only. This has been taken to indicate that at least two fundamentally different reactions are involved in the process of photosynthesis. One of them, a photochemical reaction, depends for its pace on the light intensity, and is not perceptibly affected by temperature. The other reaction is purely chemical, and is markedly affected by temperature. The effect of temperature on the rate of photosynthesis is not manifest until light of sufficient intensity is used to make the photochemical reaction exceed the chemical reaction in speed. The chemical reaction is usually designated as the "Blackman reaction," and it is supposed to be enzymatic in nature (*cf.* Warburg, 1925).

Willstätter supposes that in his leaves with low chlorophyll concentrations the photochemical reaction is slower than the Blackman reaction, even at high light intensities. If this explanation is complete and correct, it should be possible to separate the Blackman reaction from the photochemical reaction by changing the chlorophyll content as well as by changing the light intensity. Warburg (1925) gives a condensed summary of the characteristics of the Blackman reaction and the photochemical reaction. The characteristics of the former appear when photosynthesis is studied at high light intensities, of the latter when the process is studied at low light intensities. As pointed out already, the most distinctive characteristic of the Blackman reaction is its sensitivity to changes in temperature, to which the photochemical reaction is relatively insensitive. Another distinguishing feature of the Blackman reaction is that it is easily inhibited by dilute prussic acid, the photochemical reaction being relatively insensitive to the same concentrations of prussic acid.

Extremes of light intensity presumably bring about the characteristics of one reaction or the other by making the photochemical reaction slower or faster than the Blackman reaction. It should be possible to achieve the same effect by using different amounts of chlorophyll, the substance absorbing the light. The results of Willstätter and Stoll outlined above suggest that this is the case. But it seems desirable to compare their results with some obtained from more nearly comparable material.

The published data on the rate of P_{max} at different temperatures is not adequate for a comparison of

of Blackman and Matthaei and of Willstätter and Stoll are open to the objection that the temperature of the assimilating cells cannot be accurately determined in leaves. Harder (1915), Warburg (1919), and Yabusoe (1924) avoided this difficulty by working with water plants. But Harder's results are inexact because he used light intensities and carbon dioxide concentrations which were too low to cause maximum photosynthesis at the higher temperatures. His curves show that increasing either of these factors caused an increase in rate, whereas in order to study the Blackman reaction, carbon dioxide concentration and light intensity must be so high that the rate of photosynthesis is independent of small changes. Besides, Harder allowed the temperature to vary a degree or more during some readings.

The experiments of Warburg and Yabusoe on *Chlorella* are not open to these technical objections. But their readings are taken so far apart on the temperature scale that it is not possible to determine what sort of function of temperature the Blackman reaction is. Yabusoe, for instance, gives readings taken at 10°, 20°, and 30°, and finds that when the rate of photosynthesis is plotted against degrees Centigrade these three points lie on a straight line. Since the points are so few and widely separated, his conclusion that the Blackman reaction is a linear function of the temperature seems hardly justified.

It is necessary therefore to determine how the Blackman reaction of normally green cells varies with the temperature, in order to have something with which to compare the effect of changes in temperature on the rate of photosynthesis of cells poor in chlorophyll.

The measurements of rate of photosynthesis were carried out in the carbonate-bicarbonate mixture as before. At lower temperatures the dissociation constants for the salts in this mixture are smaller, and the carbon dioxide concentration is lower. However, it was found that at the lowest temperatures used this mixture still gave an adequate concentration of CO₂. The rate of photosynthesis remained independent of small changes in CO₂ tension.

A description of the apparatus for temperature control will be found in a paper by Crozier and Stier (1927, p. 503). Temperature was regulated by a sulfur dioxide compressor, activated by a mercury thermoregulator through two telegraph relays. The temperature was kept constant at any chosen temperature to within $\pm 0.05^\circ$. A fresh

sample of cells was used for each rate determination, the stock cell suspension being kept in the refrigerator. Unless the temperatures chosen were less than 2° apart, respiration was determined at every temperature. For temperatures less than 2° apart, respiration was measured at alternate temperatures, and the intermediate values were interpolated. An adapting time of 15 minutes was found to be sufficient at all temperatures.

Many determinations of rate of photosynthesis were made, at various intervals between 4° and 30° . The most satisfactory method of making the determinations was arrived at after numerous trials,

TABLE II.
Data for Curve A, Fig. 2.

Temperature	K_{O_2}	Mm ³ cells used	ΔH per 10 mm ³ cells per hour corrected for respiration	Q_{O_2}	$1/T$ absolute	Log R
$^{\circ}C.$						
4	0.59	19	23.7	13.9	0.003610	1.143
6	0.58	19	39.1	22.7	584	1.356
8	0.56	19	55	30.6	559	1.486
10	0.58	19	75	42.8	534	1.631
11	0.57	13.5	82	46.8	521	1.670
12	0.58	13.5	90	52.3	509	1.719
14	0.62	13.5	104	64.3	484	1.808
16	0.55	13.5	137	75.3	460	1.877
17	0.54	8	145	78.5	448	1.895
26	0.53	8	218	115.5	344	2.062

but a summary of all the data obtained suggests nothing at variance with the conclusions drawn from the data finally secured by the most satisfactory method.

Table II shows the rates of photosynthesis, Q_{O_2} , in cubic millimeters of oxygen per hour per 10 c.mm. of cells, for two typical runs, going from about 4° to about 25° . If these values are plotted directly against temperature centigrade, the result is not a straight line, as Yabusoe supposes, but a sigmoid curve. It is easy to see how his three points, two at the ends and one in the middle, could lie on a straight line. But it is more interesting to plot these data according

to the equation first developed by Arrhenius (1889), and which has since then been found applicable to a large number of reactions and processes, both chemical and biological.¹ If R_1 and R_2 are the rates or frequencies of a process at two temperatures, T_1 and T_2 absolute, then the Arrhenius equation states that

$$\frac{R_2}{R_1} = e^{\frac{\mu}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)}$$

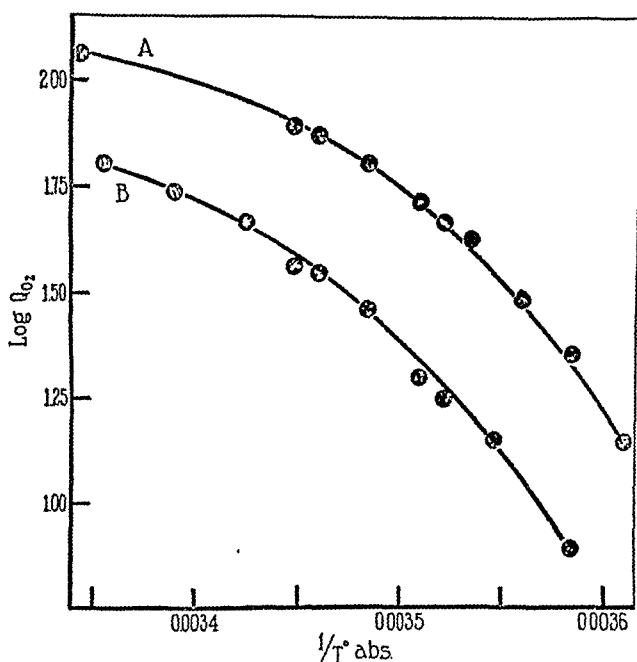


FIG. 2. Curves showing the logarithm of the rate of photosynthesis with two concentrations of chlorophyll plotted against $1/T$ absolute.

If this equation is satisfied, the rate or frequency, when plotted against the reciprocal of the absolute temperature should give a straight line whose slope is μ , a constant found to have characteristic values for certain types of reactions and processes.

Fig. 2 shows the data of Table II plotted according to the Arrhenius

¹ For examples of the Arrhenius equation applied to biological reactions and processes, see papers by Crozier and others in *The Journal of General Physiology* in recent years. Especially: Castle, 1928; Crozier and Stier, 1927; Wolf, E., 1928; Brown and Crozier, 1927.

equation. The points do not lie on a straight line, but rather on a curve which is concave downwards. The equation is apparently not satisfied. The value of μ decreases gradually from about 31,000 at low temperatures to about 16,000 at high temperatures. Hinshelwood (1926, p. 47) states that if a curve instead of a straight line is obtained from this sort of a plot, it may be concluded that the rate is being determined by the sum of the rates of two or more parallel reactions

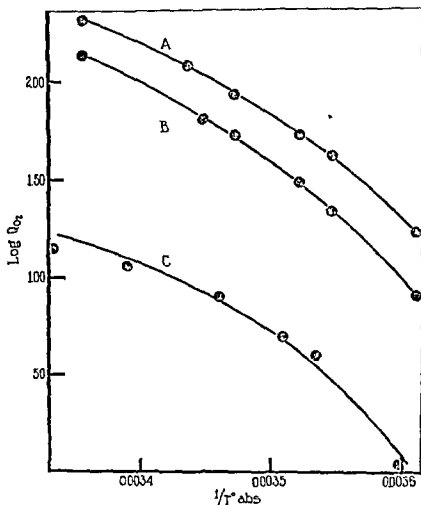


FIG. 3. The logarithm of the rate of photosynthesis plotted against $1/T$ absolute, with three different chlorophyll concentrations.

having different temperature characteristics. He cites as an example the case investigated by Norrish and Rideal (1923) of the combination of sulfur and hydrogen. This takes place in a gas reaction, for which $\mu = 52,000$, and a surface reaction on the walls of the container, for which $\mu = 26,000$. Hinshelwood's plot of the data of Norrish and Rideal shows a curve which is concave upwards. It is difficult to see

how this could be otherwise, since the higher the temperature the more the reaction with the higher temperature characteristic will predominate. Since the temperature characteristic for photosynthesis *decreases* with rising temperature, it seems unlikely that the case can be explained in the same way as that of Norrish and Rideal.

TABLE III.
Data for Fig. 3.

	Temperature	1/T absolute	Log R
	°C.		
Curve A $\epsilon = -0.101$	4	0.003610	1.230
	9	546	1.622
	9	546	1.592
	11	521	1.728
	15	472	1.932
	18	436	2.093
	25	356	2.318
Curve B $\epsilon = 0.048$	4	610	0.908
	9	546	1.344
	9	546	1.335
	11	521	1.484
	15	442	1.726
	17	448	1.809
	25	356	2.133
Curve C $\epsilon = 0.016$	5	597	0.041
	10	534	0.602
	12	509	0.699
	16	460	0.898
	18	436	1.029
	27	333	1.149

Crozier (1926) in a paper on growth in relation to temperature shows several curves of the logarithm of the rate plotted against $1/T$. They show a uniform tendency to be concave downward, which he suggests is consistent with the autocatalytic nature of the process of growth. If a monomolecular reaction is catalyzed by its end-product, then raising the temperature will affect not only the primary reaction, but also the catalysis of the reaction by its end-product. If the temperature characteristics of these reactions differ numerically, then the

curve of the logarithm of the rate of the entire process plotted against $1/T$ must necessarily be concave downward. It is therefore suggested that the process of photosynthesis may involve an autocatalytic reaction, in which the temperature characteristic for the catalysis differs from the temperature characteristic for the simple reaction.

It is of interest to know whether the shape of the temperature curve will be altered by reducing the chlorophyll content per unit volume of cells. As mentioned above, changing the light intensity does produce a change in the shape of the temperature curve. Fig. 3 shows three curves made with three sets of cells differing widely in chlorophyll content. The coordinates of the points in Fig. 3 are given in Table III. For curve A, $\epsilon = 0.101$, for curve B, $\epsilon = 0.048$, and for curve C, $\epsilon = 0.016$. Curves A and B are strictly comparable. Curve C was made from a culture of a different series. The highest point on curve C was obtained at 27° , a temperature higher than was ordinarily used. Temperatures above 25° are injurious to cells suspended in the carbonate mixture. Their capacity for photosynthesis is impaired after short exposures. This probably accounts for the low position of the top point of curve C.

All three curves have about the same shape, showing that the effect of temperature change on the rate of photosynthesis is the same over this range of chlorophyll concentrations. Decreasing the rate of photosynthesis by lowering the chlorophyll content does *not* produce the same change in this characteristic of the process as decreasing the rate by lowering the light intensity.

III.

The Effect of Prussic Acid on the Rate of Photosynthesis with Different Concentrations of Chlorophyll.

As mentioned in the preceding section, the Blackman reaction is characterized by sensitivity to dilute prussic acid, as well as to changes in temperature. Warburg (1919) found that with decreasing light intensity this sensitivity to prussic acid vanished. In view of the response of photosynthesis to changes in temperature at low concentrations of chlorophyll, it cannot be expected that inhibition of the process by prussic acid will decrease with decreasing chlorophyll content.

Experiments were made by adding free HCN to cells suspended in a solution containing the same salts as the culture medium (KH_2PO_4 , KNO_3 , MgSO_4), or by adding KCN to cells suspended in the carbonate mixture. Both methods produced the same result. The experiments in the carbonate mixture are simpler, and they alone need be described.

When potassium cyanide is added to the carbonate mixture, the concentration of free prussic acid will depend on the amount of cyanide added, on the temperature, and on the hydrogen ion concentration of the solution. Following Warburg, we shall write

- C = total cyanide concentration,
 H = hydrogen ion concentration,
 K = dissociation constant of HCN at the temperature of the experiment,
 X = concentration of free HCN

Then,

$$X = \frac{C \times H}{H + K} \quad (1)$$

Warburg sets $H = 10^{-9.36}$ at 25°C . for the carbonate mixture used in these experiments (Warburg's mixture No. 9). No change in pH could be detected with indicators over a wide range of temperature, so the value at 20° cannot differ appreciably from this figure. Landholt-Börnstein (1912, p. 1138) gives $K = 4.7 \times 10^{-10}$ at 18° and $K = 7.2 \times 10^{-10}$ at 25° . Interpolating, $K = 5.4 \times 10^{-10}$ at 20° . Substituting these values in (1), we find that at 20°

$$X = \frac{C}{2.25}$$

In these experiments a cyanide concentration of 2×10^{-5} moles per liter was used. This gave a prussic acid concentration of about 9×10^{-6} normal.

In each experiment two vessels were used, one without cyanide, the other containing $\text{KCN} = 2 \times 10^{-5}$ normal, each vessel containing the same quantity of cells. Thin suspensions were used, and high light intensity was obtained from a 300 watt lamp, immersed in the thermostat directly below the vessels. The light of the tungsten ribbon lamp previously used could not be satisfactorily spread out over two vessels.

Table IV gives the results of one experiment. As the chlorophyll concentration decreases from 0.831 to 0.037, the inhibition by HCN increases from 25 to 40 per cent. Lowering the chlorophyll concentration thus reverses the effect of lowering the light intensity. The sensitivity to prussic acid *increases* instead of decreasing. Photosynthesis continues to show the characteristics of the Blackman reaction when the chlorophyll content per unit volume of cells is lessened, although when the light intensity is cut down the process loses these characteristics. The immediate effect of decreasing either the light intensity or the chlorophyll content is to decrease the rate of photosynthesis. It is supposed that decreasing the light intensity

TABLE IV.

The Effect of Prussic Acid on Photosynthesis at Three Different Concentrations of Chlorophyll.

c	HCN concentration	Mm. ³ O ₂ produced	Inhibition by HCN
			<i>per cent</i>
0.037	0	93.1	40
	9×10^{-6} N	56.2	
0.060	0	122.0	32
	9×10^{-6} N	82.7	
0.083	0	171.5	25
	9×10^{-6} N	129.5	

alters the characteristics of the process of photosynthesis because it cuts down the rate of the photochemical reaction while leaving the Blackman reaction unaffected. The Blackman reaction, then, cannot go faster than the photochemical reaction, and the characteristics of the former cease to appear when the process is studied as a whole. Decreasing the amount of chlorophyll present must also cut down the rate of the photochemical reaction, because chlorophyll is the agent which absorbs the necessary energy for that reaction. But because the characteristics of the Blackman reaction still remain in evidence, it must be supposed that chlorophyll plays a part in that reaction as well as in the photochemical reaction. Unless the concept of photo-

TABLE V.
Detailed Data for Fig. 1.

Absorption data	Filter combination.....	5-10	5-50	5	10	25	50	75	No filter
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	
	Relative intensity.....	2	10	20	40	100	200	300	400
	Log I.....	0.301	1.000	1.301	1.602	2.000	2.301	2.477	2.602
Amount cells extracted = 52 mm. ³ $\phi_1 = 49.3^\circ$ $\phi_2 = 40.4^\circ$ ϵ per 10 mm. ³ = .026	No. mm. ³ cells used	15.5	15.5	15.5	15.5	15.5	15.5	15.5	15.5
	K_{O_2} at 20°C.	0.54	0.55	0.53	0.53	0.56	0.60	0.55	0.54
	ΔH mm. per hr. per 10 mm. ³ cells	-10.5	-7.8	4.7	14.7	42.0	42.5	42.0	42.0
	Correction for respiration, mm. ³ O ₂ per hr.	-10.0	-10.0	-10.0	-10.0	-10.0	-10.0	-10.0	-10.0
	Q_{O_2} , mm. ³ O ₂ per hr.	4.3	5.7	12.5	17.8	33.5	35.5	33.1	32.7
Amount cells extracted = 38 mm. ³ $\phi_1 = 57.5^\circ$ $\phi_2 = 33.4^\circ$ ϵ per 10 mm. ³ = .099	No. mm. ³ cells used	16	16	16	16	16	8	8	8
	K_{O_2} at 20°C.	0.53	0.54	0.55	0.53	0.55	0.60	0.54	0.55
	ΔH mm. per hr. per 10 mm. ³ cells	-15	6.8	26.0	61.0	145	160	187	186
	Correction for respiration, mm. ³ O ₂ per hr.	-10	-10	-10	-10	-10	-10	-10	-10
	Q_{O_2} , mm. ³ O ₂ per hr.	2.1	13.7	24.3	42.4	90.0	106	111	112

synthesis as a process involving a Blackman reaction and a photochemical reaction is discarded, it seems to me that this is the only logical conclusion to be drawn from the results presented in this paper.

SUMMARY.

1. Photosynthesis reaches its maximum rate at about the same light intensity over the whole range of chlorophyll concentrations studied.

2. Over this range the process shows the same relationship to temperature. The value of the temperature characteristic decreases gradually as the temperature rises.

3. The rate of photosynthesis is more depressed by prussic acid the lower the chlorophyll concentration.

4. These results are interpreted as indicating that photosynthesis possibly involves an autocatalytic reaction, and that chlorophyll must play some part in the process in addition to its rôle in the absorption of light.

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RESPIRATION AND GEOTROPISM IN VICIA FABAE. I.

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The nature of geotropic responses in roots and stems is a problem whereon many people have worked for a long period. Since the first indications of such responses, by J. B. Denis (1673), up to the present time, the number of papers and memoirs dealing with contributions to the theory of this phenomenon has grown to a large figure (about 1,400).¹ And still, we know very little of the intimate character of the processes starting the response and occurring during its execution. We know a little about certain time relations binding together a few variables that we can recognize as significant in the experiments, but we are at present unable to say much about the mechanism of these relations. Even certain points which may be of primary importance seem not to have been touched (*e.g.*, work necessary for curvature, liberation of heat, and the like). Furthermore the advance in physical chemistry in the last 20 years and the new tendencies in general physiology born during the last decade enable one to consider this problem from new viewpoints. It gives also the opportunity to look at things known for a long time with new ideas in mind, and perhaps to gain a clearer picture of the geotropic response.

In the first part of this work we will deal mainly with the respiration of *Vicia faba* seedlings in a state of rest. We will follow also the variations of the geotropic reaction time with change of temperature. From the type of variation so found we may derive indications as to the kinetics of the underlying processes. The second and third parts will deal with variations in the rate of respiration during geotropic response, and with other measurable changes in the responding tissues.

¹ For a careful and complete bibliography of the subject the reader is referred to the work of Miss Christiansen, *Bibliographie des Geotropismus*, *Mill. Inst. allg. Bot.*, 1917, and following years.

I.

Conditions of Experimentation.

A.—The device used for measuring the quantities of CO_2 excreted by a single plant or a single root did not depart from the standard methods by more than a few minor modifications. Special care was given to the control of each step in the procedure. A supply of pure air (without even traces of CO_2) was led to a glass respiration chamber immersed in a water thermostat whose temperature was kept constant to within 0.01°C . for any one run. After leaving the respiration chamber the air was bubbled through a Kunz absorption flask containing a concentrated solution of KOH, where all the CO_2 carried over from the plant could be absorbed. Weighings at intervals of 15 or 30 minutes gave the quantities of CO_2 excreted by the plant in the corresponding periods. All the procedure was carried on in a dark room; dim non-actinic red light was the only source of illumination used, and even then for the shortest durations possible.

As anyone will see, the method employed is one very widely used; nevertheless certain details have to be given as to how it was treated. The air which is supplied to the respiration chamber must be pure: it may not contain fumes or traces of H_2S , SO_2 , NH_3OH , illuminating gas, etc., in addition to the normal content of CO_2 of the atmosphere. In the apparatus used in these sets of experiments the air was pumped from outside the building, by means of two glass bottles arranged in such a way as to allow a given and constant amount of water to flow from one to the other and thus producing suction. Before entering the metallic piping, absolutely leakproof, which was leading it to the thermostat, the air was freed from dust by passage through 35 cm. of cotton tightly pressed in an absorption tube; furthermore a concentrated solution of mercuric chloride and a 15 per cent solution of H_2SO_4 were freeing the air from the traces of the common gases found. The partially purified air was now passed through a long train composed of (1) three absorption tubes containing soda-lime granules (size No. 4) between cotton plugs, (2) three gas washing bottles with concentrated NaOH solution, (3) one long tube containing a dilute solution of NaOH (4) a tube 45 cm. high containing, to a depth of 35 cm., glass beads and distilled water freed from CO_2 and in which the current of air was introduced under the beads and forced through them, (5) a 12 m. length of copper tubing containing in the two lower spires a small quantity of distilled water. Numbers 2, 3, 4 and 5 were all immersed in the water thermostat where the experiment was performed so as to be in perfect temperature equilibrium. The junctions between the different parts of the apparatus were all glass-to-glass; the rubber tubings, used only where ground-glass joints would have been unpracti-

cal, were heavily coated with a celluloid cement. There was supplied in this way a current of air which, when analysed with the Haldane apparatus, did not show traces of CO_2 or of any of the objectionable gases of a city atmosphere. It was furthermore always saturated with water vapor at the temperature of the thermostat.

The respiration chamber consisted of a 250 cc. gas wash-bottle with fairly wide mouth and long and well ground head piece. The glass tube which normally leads the gas in was bent to one side and used as a support for the plant. This device had the advantage of being very easy manipulated, maintained perfectly sterile, and was possible to place it inside the thermostat in any position, so that geotropic excitation could be applied to the organism studied merely by turning the bottle on its side while all the other conditions remained unchanged. The smallness of the respiration chamber had the advantage of allowing a very easy flushing of all gases liberated in it. The absence in the same chamber of filter paper lining or cotton pads, moistened with water to keep the atmosphere saturated with water vapor (as sometimes used; *cf.* Kuyper, (1910) p. 144; and Fernandes, (1923) p. 121), prevents all solution of CO_2 in this water. The coefficient of partition of CO_2 between water and air is so much more in favor of water that one may expect a certain amount of liberated CO_2 to be held in aqueous solution. That this solution follows the same law as the excretion of CO_2 , in relation to temperature, is still to be examined. But in any case there is here a source of serious error which is eliminated by the scheme here adopted. To such cause may be attributed the apparent better constancy shown by the figures of Kuyper (p. 165) in his third experiment, in contrast to his two first experiments performed without addition of water in the respiration chamber. It is also to be noted that the figures corresponding to his third experiment are lower, on the average, than in the other sets.

Furthermore, the use of a Pyrex glass chamber makes one sure that no disturbing reaction in which the copper or aluminium used in some apparatus (*cf.* Kuyper, p. 144), will take place. One may remember the extreme toxicity of copper, especially in soluble salts, and the not less toxic effect of aluminium salts (Szűcs, 1913).

A valve was placed between the respiration chamber and the absorption flask to enable us to cut the latter out of the circuit without disconnecting any part of the apparatus. This device was used always at the start of an experiment when the whole circuit had to be flushed and cleaned for $\frac{1}{2}$ hour by a current of pure air, without being obliged to absorb all the possible CO_2 present in the absorption flask.

The pump consisted of two 2 liter flasks with a lower tubulure connected by a rubber tubing. The upper neck opening of flask No. 1 (which was always kept at the same place) was connected through a soda-lime tube to the absorption flask; a stop-cock and a Mohr screw clamp were placed on this connection. The same rubber stopper in the neck of flask No. 1 was fitted also with a small soda-

lime tube normally closed by a stop-cock. When the 2 liters of water had drained from No. 1 to No. 2, it was a simple matter to refill bottle No. 1: the stop-cock on the connection with the absorption flask was closed, the one on No. 1 opened, bottle No. 2 was lifted from the lower position (90 cm. below No. 1) to the upper position (about 75 cm. above No. 1) and the refilling of No. 1 was readily done. To start over again the stop-cocks were turned to their first position. The Mohr clamp was adjusted once for all to obtain a flow of 2 liters of air in 30 minutes; the rate of passage of bubbles through the Kunz absorption flask was in this way adjusted to obtain total absorption of CO_2 in one passage, as was ascertained by tests where a second flask was placed after the normal absorption flask.

The absorption medium in the Kunz flask was a solution of KOH (1 to 1). This solution absorbed naturally also the small amount of water vapor saturating the air current supplied to the respiration chamber; this quantity, which was remarkably constant even over long durations, was determined by a few runs before and after the plant was under observation. It was then a simple matter to correct the readings for the absorption of water.

We have discarded the titrimetric method of estimation of CO_2 absorbed by a solution of $\text{Ba}(\text{OH})_2$ with a final titration using phenolphthalein as indicator, on account of a cause of error which seems to have been overlooked. The solubility product of BaCO_3 is very small only as long as the solution is strongly alkaline. When the neutralization is proceeding, the equilibrium is not the same any more and a part of the CO_2 may be driven away in a gaseous state out of the solution. Furthermore, as the redissolution of the precipitate of BaCO_3 is rather slow, the titration end-point will vary with the speed of addition of the acid. Further still, phenolphthalein has a turning point which is fairly above neutrality.²

B.—The plants used in these experiments were young seedlings of *Vicia faba* (Broad Windsor beans), grown in sterile sawdust, in a dark thermostat, at a temperature of 20°C . The seeds had been soaked for 24 hours in running water, disinfected quickly with a 1 per cent solution of formaldehyde or a 3 per cent solution of H_2O_2 , rinsed in sterile distilled water and planted immediately. The seedlings were used when the roots reached a length of 4 to 5 cm. (*i.e.*, in the 4th day) and the samples chosen were with perfectly straight roots. The respiration chamber in which the plant was placed was disinfected before and after each run with 94° alcohol and 3 per cent solution of H_2O_2 , and rinsed with sterile distilled water. Decay or drying never arose during the runs, and on three occasions the plant was kept for 3 days in the chamber without showing any sign of disease. This indicates that the maintenance of a plant in the respiration chamber for dura-

² These points will be reviewed in a note dealing with CO_2 titration.

tions of 24 to 36 hours did not bring into play any uncontrolled factor. In the series of experiments where only parts of the plants were used (root or intact plant attached to a small portion of the cotyledons), all the dissections were made under the red light illumination already mentioned, care being taken also to keep the plant in a normal position during the whole treatment. The cut surfaces were covered immediately with strips or small pieces of cover-glass slightly coated with neutral vaseline. All the manipulations were done as quickly as possible and under aseptic conditions.

As it was thought that these experiments would require a fairly long time before completion, a large stock of seeds was taken so as to have material as homogenous as possible. Several tests performed at different intervals showed that the keeping of the seeds in jars where evaporation was very much prevented did not bring appreciable source of error. The rate of excretion was found to be constant for seeds kept for more than 8 months in the condition described.

C.—In all the experiments care was taken that thorough equilibrium of temperature was attained before beginning the measurements of CO_2 excretion. The plants were allowed to stay for 30 minutes at least in the respiration chamber, normally ventilated with pure air, before the current of air was switched through the absorption flask.

A very extensive series of published and unpublished cases has shown that it is absolutely necessary to have a perfect control of temperature for the thermostat *used in such experiments*. *It must not only be possible to keep any given temperature for any length of time, but also to pass quickly from one temperature level to another and to obtain then the same ease and constancy of control.* Furthermore, the thermostat used for biological purposes must be one where the successive temperatures obtainable must be very close one to the other and not only at a few points distributed more or less regularly along the thermometric scale.³

The fluctuations of any air thermostat exclude completely this type as too inaccurate. We have used a water thermostat of about 100 liters capacity. A lead coil was supported inside the tank, at the middle of its height, the two ends providing an inlet and an outlet for a current of cold water. This current was regulated so as to cool rather quickly the water of the thermostat which was kept constantly stirred by an electrically driven stirrer. On the other hand, an electric heating unit (with a very large surface and a small mass) was placed on one side of the tank in the whirlpool made by the eddy-currents of the stirrer. The distribution of heat was so obtained in a very efficient way. The heating unit was working

³ About thermostats *cf.* Crozier and Stier (1926-27).

on A.C. at 110 volts and was thrown in or out of action by a high resistance relay placed in a circuit with two to four dry cells and a large mercury thermoregulator. The volume of the mercury and the diameter of the capillary of the thermoregulator were chosen to give easily a regulation within $\pm 0.01^{\circ}\text{C}$. The temperature was read after each observation made, *i.e.* every 15 or 30 minutes. This arrangement provided a thermostat which was run without any trouble for days and even weeks.

D.—As it is impossible to have two lots of seedlings having all characteristics identical (length of root, weights of different parts, *etc.*), one may wonder to what common base the amounts of CO_2 excreted must be reduced. Will it be necessary to use the *individual* as unit, or *unit weight* (fresh or dry), or *unit surface*? The first may be discarded as being too irrelevant; the latter also on account of the diffi-

TABLE I.

The Relative Proportions of Fresh and Dry Weight of Seedlings of Vicia faba with Their Ranges of Absolute Variation.

		range
Mean fresh weight.....	$5.6450 \pm 0.173 \text{ gm.}$	5.012 to 6.460
Mean dry weight.....	$1.8557 \pm 0.057 \text{ gm.}$	1.4419 to 2.1714
Mean ratio $\frac{\text{Dry weight}}{\text{Green weight}}$	$32.2 \pm 1.0 \text{ per cent}$	

culty of ascertaining with any precision the surfaces involved. The only basis that remains is then the weight. But shall we use green weight or dry weight? In fact, as will be seen from the table given (see Table I), the variations of green and dry weights are rather small for the samples used, and as the ratio of dry weight to green weight is fluctuating in still much narrower limits, we will refer all CO_2 excreted to the fresh weights of the seedlings.

It must also be noted that to a very good approximation there is for *Vicia faba* a direct relationship between the CO_2 excreted and the weight of the seedling. It seems to us therefore perfectly proper to reduce our data to a rate of excretion per standard weight of plant and per unit interval of time.

Finally, in the cases where total seedlings or parts of seedlings were to be compared, we have been able to reduce all the quantities found

to the same standard, taking into account that if at any given temperature the total CO_2 excreted by a complete seedling is 100 per cent, the cotyledons give 67 per cent and the plantule gives 33 per cent. Of 33 per cent the stem excretes 18 per cent and the root 15 per cent.

The constancy of these figures is remarkable as they have been found on a number of occasions. In fact this constancy allowed us, in the great majority of cases, to use intact seedlings, which were more easy to handle and which also fitted better our principle of "no interference" with the normal activity of the organism than would the dissecting or removing of parts of it.

II.

The Resting Metabolism.

A review of the literature concerning the relation of respiration of seedlings to temperature is rather simplified by the small number of papers published on this matter. This situation is in fact even remarkable when one takes into account the importance of the processes involved. It must be said, also, especially for most of the older work, that the lack of precision in the experimental conditions (control of temperature, scarcity of the points chosen on the thermometric scale) obliges one, if not to discard practically all these data, at least to consider them for the most part as simply indications.

The first work where a fair constancy of temperature and temperature control are found, are in the papers by Rischawi (1877), Kreusler (1887-88) and Clausen (1890).⁴ But it must be said that these authors were more interested in the determination of the "optimum" temperature for respiration than in the exact relation of respiration to temperature. So we find that these papers pay special attention to the higher temperatures and, as was pointed out by Kuyper, the differences existing between these three sets of results can be ascribed to several causes of which one is the treatment of the organisms at high temperature. Clausen especially seems not to have paid enough at-

⁴Nevertheless in Kreusler's work he notes that differences in temperature between two parts of his respiration chamber were 1° to 2° , and in unfavorable cases even 3° to 4° ; he also says "so hat das gewiss nicht viel zu bedeuten" (*Landw. Jahrb.*, 1890 xix, 651).

tention to the effect of maintaining plants for a duration of 2 to 3½ hours at temperatures of 35°C. and even higher.

The most elaborate and careful work yet published is that of Kuyper (1910). After having pointed out several mistakes of his predecessors in this line of investigation, he proceeded to show that one of the chief causes of error was precisely that of keeping the plants for long duration at high temperature. The prehandling of the plant determines to a certain extent the results obtained, and in the interpretation of the data a time factor must be taken into consideration. But, as with his forerunners, he also is mostly interested in high temperatures; he attempted to test the applicability of Blackman's theory of limiting factors to normal respiration (cf. p. 139). However, even if his interest lies in the upper part of the thermometric scale, the range 0° to 25°C. is considered with care.

The results of his work (done mostly on *Pisum sativum*, *Lupinus luteus* and *Triticum vulgare*) showed that the respiration rates studied for periods of 6 hours show a very great constancy up to 10°C.; for temperatures up to 20°C. one finds first an increase followed by a decrease in the rate of excretion; for still higher temperatures up to 40°C. the same thing holds true but much more exaggeratedly. The drop in rate seems to follow a logarithmic curve. The critical temperature where one type of respiration (constant) passes to the other type (decreasing) seems to depend on the nature of the reserve substances; being higher for amylaceous reserves, lower for protein reserves. Van't Hoff's rule is valid for *Pisum* and *Triticum* in the range 0° to 20°C., for *Lupinus* up to 25°C. Q_{10} has a value between 2 and 3.

Change in temperature does not "activate" the respiration. The work of Kuyper shows very clearly the existence of critical temperatures above which the phenomenon studied seems to take a new course. This is a point of special interest to us, to which we will have the occasion to come back later. As to the question of applicability of Blackman's theory, as well as about the significance of the Q_{10} obtained, one may differ in opinion. As a whole, this work is the foremost and the data can be accepted as the most precise yet published.

In the experiments here described, all performed with single, entire seedlings, we chose the following temperatures on account of the fact that they cover the range of the thermometric scale where normal geotropic responses could be gotten. These measurements of CO₂ ex-

TABLE II.

CO₂ Excretion by Sets of Five Seedlings at Each Temperature, with the Reductions to 100 Gm. Fresh Weight Per Hour, and the Variability.

Temperature	Weight	CO ₂ per seed per hour	Mean	CO ₂ per 100 gm. per hour	Mean	Deviation from mean	Mean deviation	Mean deviation as percentage of mean
°C.	gm.	mg.		mg.				per cent
7.5	5.10	1.15	1.29	22.55	23.11	0.56	0.426	1.84
	5.31	1.20		22.60		0.51		
	5.51	1.27		23.14		0.03		
	5.88	1.40		23.81		0.70		
	6.10	1.43		23.44		0.33		
10	5.20	1.57	1.72	30.29	30.154	0.13	0.085	0.28
	5.54	1.67		30.23		0.07		
	5.65	1.70		30.09		0.06		
	5.97	1.80		30.15		0.006		
	6.23	1.87		30.01		0.14		
12	5.17	1.92	2.15	37.14	37.24	0.10	0.082	0.22
	5.36	2.00		37.31		0.07		
	5.70	2.13		37.37		0.13		
	6.18	2.30		37.22		0.02		
	6.46	2.40		37.15		0.09		
15	5.01	2.48	2.73	49.50	49.24	0.26	0.136	0.27
	5.29	2.60		49.15		0.09		
	5.80	2.85		49.14		0.10		
	5.98	2.95		49.33		0.09		
	6.17	3.03		49.10		0.14		
17.5	5.11	3.27	3.57	63.99	63.75	0.24	0.198	0.31
	5.45	3.45		63.30		0.45		
	5.65	3.60		63.72		0.03		
	5.78	3.70		64.01		0.26		
	6.04	3.85		63.74		0.01		
20	5.27	4.35	4.72	82.54	83.10	0.56	0.38	0.46
	5.60	4.65		83.03		0.07		
	5.69	4.75		83.48		0.38		
	5.70	4.77		83.68		0.58		
	6.16	5.10		82.79		0.31		
22.5	5.23	5.15	5.56	98.47	98.77	0.30	0.782	0.79
	5.40	5.40		100.00		1.23		
	5.50	5.45		99.10		0.33		
	5.92	5.75		97.13		1.64		
	6.08	6.03		99.18		0.41		

TABLE II—*Concluded.*

Temperature	Weight	CO ₂ per seed per hour	Mean	CO ₂ per 100 gm. per hour	Mean	Deviation from mean	Mean deviation	Mean deviation as percentage of mean
°C.	gm.	mg.		mg.				per cent
25	5.18	5.80	6.36	111.96	112.86	0.90	0.64	0.57
	5.32	6.05		113.72		0.86		
	5.70	6.40		112.28		0.58		
	5.88	6.68		113.60		0.74		
	6.12	6.90		112.74		0.12		

cretion were indeed collected in order to study the kinetics of the geotropic curvature. The temperatures were: 7.5°, 10.0°, 12.0°, 15.0°, 17.5°, 20.0°, 22.5°, 25.0°. Higher temperatures were disregarded for the present, for reasons which will be explained later. The normal duration of a single experiment ranged from 6 to 12 hours, during which estimations of the CO₂ excreted were made every 15 or 30 minutes.

For each plant used, the green weight was determined immediately after the run, care being taken also to determine separately the green weight of the cotyledons, stem, and root. These different parts of the seedlings were then dried for 4 to 6 hours at 105°, and after cooling the dry weight was obtained also. This procedure gave us the following results (see Table I).

The distribution of the variations of weight is quite symmetrical. For this reason, when the CO₂ output was obtained for a known weight of plant, the excretion value was reduced to a standard weight of plant of 5.65 gm.

Contrary to what previous observers have claimed about the non-direct relationship between CO₂ output and the weight of the plant excreting it, we have found a very good agreement between the two quantities, as will be seen from Table II.

Another point which seems of interest is that, contrary to what Kuyper (p. 166 and ff.) and Fernandes (1923, p. 170) describe, we could not find that the rate of excretion was changing with time. Outside of the normal, small variations in the readings (which never reached three times the small experimental error), the rate of excretion

was found to be constant, for the periods considered. It must be said that in Kuyper's work the means of appreciation of constancy in the rate of excretion are rather vague. He considers, for instance, that for the temperatures 0° , 5° and 10° the respiration is constant; on the contrary, for the temperatures 15° and 20° the constancy is not the rule. His Table I (p. 166) is intended to illustrate the foregoing. Let us consider it more closely (see Table III).

From the figures given in Table III he concludes that—as the variations lay well in the limits of 1 mg.—the rates at 0° , 5° and 10° are

TABLE III.

Kuyper's Data for Excretion of CO_2 in Successive Hours. The Two Last Columns Are Added to Show the Mean Deviations, and the Mean Deviation as a Percentage of the Mean.

Temperature	1st hr.	2nd hr.	3rd hr.	4th hr.	5th hr.	6th hr.	Mean	Mean deviation	Mean deviation as percentage of mean
$^{\circ}\text{C.}$									per cent
0	4.0	4.0	3.6	4.0	3.8		3.9	0.16	4.10
5	6.7	6.0	5.6	5.7	5.8		6.1	0.38	6.20
10	12.0	11.2	11.1	11.4	11.4	11.3	11.4	0.20	1.75
15	18.6	19.0	19.4	19.4	20.0	20.2	19.4	0.36	1.86
20	28.6	30.3	30.3	30.6	30.4		30.0	0.60	2.00
25	43.3	42.4	42.2	49.7	40.7	40.9	42.0	0.76	1.81
30	51.7	50.9	52.2	53.6	53.5	53.5	52.6	0.96	1.70

constant. But we may object that here, the absolute variation is not a fair indication of variability. The mean deviation as a percentage of the mean would be the index to rely on. The two last columns of Table III (added by us) give a clear indication of a good constancy over the whole range here considered, except at 5° . And even for this last temperature, this does not point necessarily to a lack of *precision* in the measurements. We must give to all the figures the same weight; in such case this deviation may become indicative of some other factor coming into play. This is precisely the type of variation that we get when the temperatures considered are "critical temperatures" for the process involved (*cf.* Crozier, 1924, *a*; Crozier and Federighi, 1924-25). We may add that the second maximum

in these data, reached at 20° , is obtained precisely in a region where a second critical point for this phenomenon is known to be located. The relative smallness of this second maximum can be attributed probably to the fact that we have no data for some intermediate temperatures between 20° and 25° ; the critical temperature is in fact, around 21° (cf. later), and the value 2.0 per cent may be below the actual

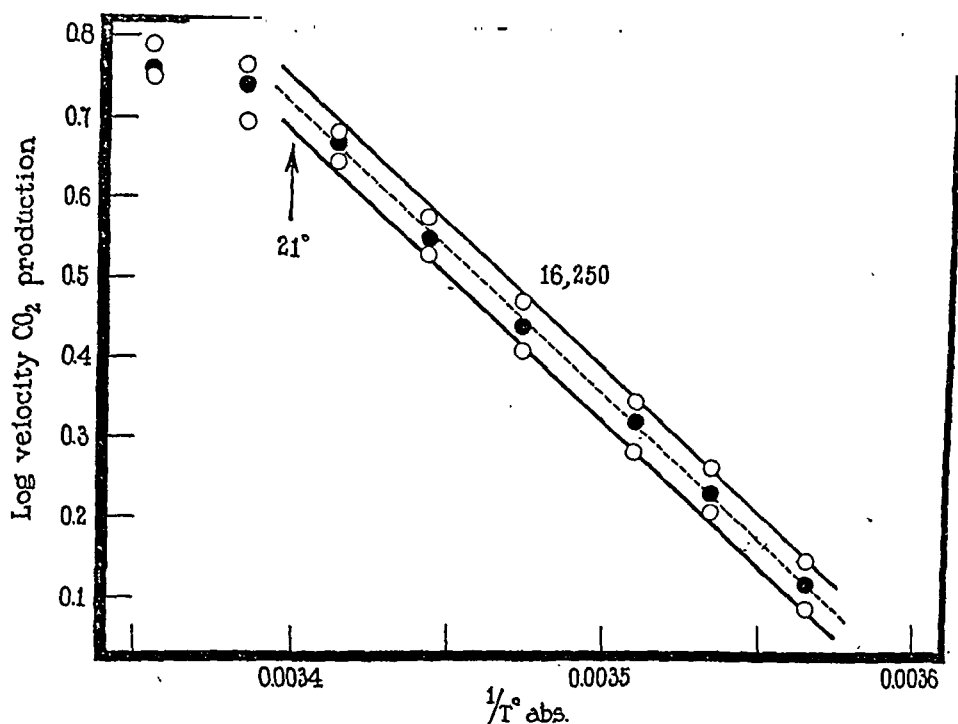


FIG. 1. CO₂ production by seedlings of *Vicia faba*. The broken line corresponds to the line best fitting the data in Fig. 3, the two heavy lines describe the band of variations of the data from which the averages were made, the light circles represent for each temperature the extreme variations. It should be noted also how much the latitude of variation is increased around the upper critical temperature.

maximum of deviation. As has been shown on several occasions by Crozier (1924, *a*), and Glaser (1925-26), the latitude of variation of observations is often much larger at critical temperatures than at any other places in the temperature range (Fig. 1). In this respect, the observations of Kuyper show a remarkable parallelism with ours, if we consider a measure of variability in the rate of excretion to be given by the mean deviations as a percentage of the mean, since in

both cases we find the variability to be maximum at the same temperatures (Fig. 2). As the two methods of measurement and the material used were totally different, the fact that we get such a parallelism points once more to the importance of these critical temperatures. It shows also the absolute necessity, when a biological process is investigated from the viewpoint of its variation with temperature, to have a large number of observations scattered over the whole range

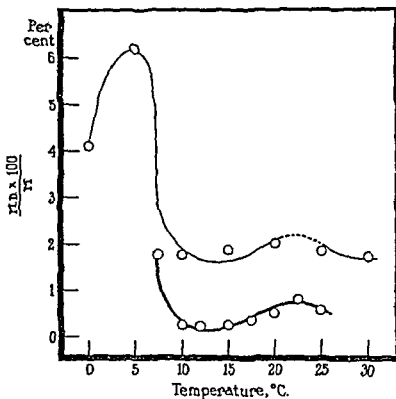


FIG. 2. The relation of the mean deviation as a percentage of the mean, in series of determinations of CO_2 excretion by seeds of *Pisum* (Kuyper: CO_2 mg. per 100 gm. per hour) and of *Vicia faba* (CO_2 mg. per 100 gm. per hour), to temperature, in Kuyper's data (light line) and personal data (heavy line). The absolute values of these deviations are shown also.

studied (and not grouped at 5° intervals, as has so often been the case previously). The summary of most of the data so obtained will be found in Table IV,⁶ where all the weights of CO_2 excreted per hour have been reduced in terms of the standard seedling of 5.65 gm.

⁶ The slight discrepancies found between Table IV and the figures given in Table II are due to the fact that in the latter case five samples, evenly distributed over the range of seedling weight, were taken. Table IV on the contrary takes into account all the experiments performed.

The measurements so obtained can be utilized in several ways. We may apply to them the method A. Kanitz (1915) used in his book "Temperatur und Lebensvorgänge;" that is to say, we may determine, as has been done for a very large variety of chemical processes, the temperature coefficient Q_{10} for a rise in temperature of 10°C . A good many protoplasmic activities have it in common with a large number of chemical reactions that their velocities are doubled or tripled for a rise in temperature of 10° , at ordinary temperatures. But it may be objected that the Q_{10} is a very inefficient way of characterisation of processes, as it is dependent

TABLE IV.

CO₂ Excretion Per Hour by Standard Seedling (i.e., Reduced to Weight 5.65 Gm.) of Vicia faba, at Different Temperatures. These Figures Are Plotted in Fig. 3.

The P.E. of the mean and the P.E. as a percentage of the mean are also given for CO_2 excretion by 100 gm. of seedlings at different temperatures; the respiration computed on this basis gives the same value for μ . The variability ($\text{P.E.}/M \times 100$) is a constant percentage of the mean, between critical temperatures.

Temperature °C.	CO ₂ per standard seed per hour mg.	Mean CO ₂ per 100 gm. seeds per hour	
		mg.	P.E./M \times 100 per cent
7.5	1.30	23.20 \pm 0.12	0.53
10.0	1.705	30.14 \pm 0.09	0.32
12.0	2.104	37.30 \pm 0.105	0.28
15.0	2.78	49.32 \pm 0.15	0.29
17.5	3.60	63.67 \pm 0.15	0.24
20.0	4.69	82.98 \pm 0.21	0.26
22.5	5.56	98.82 \pm 0.37	0.37
25.0	6.38	112.64 \pm 0.20	0.18

on the range of temperature chosen (*cf.*, for instance, Crozier, 1924-25 *b*, 1925-26 *c*). Kuyper had found for his data the following values of Q_{10} :

$\frac{A_{10^{\circ}}}{A_{0^{\circ}}} = 2.4$, $\frac{A_{15^{\circ}}}{A_{5^{\circ}}} = 3.1$, $\frac{A_{20^{\circ}}}{A_{10^{\circ}}} = 2.8$ for one set of experiments (average about 2.8); $\frac{A_{23^{\circ}}}{A_{13^{\circ}}} = 2.2$, $\frac{A_{25^{\circ}}}{A_{15^{\circ}}} = 2.0$ for a second set of experiments (average about

2.1). For our own data we find $\frac{A_{17.5^{\circ}}}{A_{7.5^{\circ}}} = 2.78$, $\frac{A_{20^{\circ}}}{A_{10^{\circ}}} = 2.75$, $\frac{A_{22.5^{\circ}}}{A_{12^{\circ}}} = 2.6$,

$\frac{A_{25^{\circ}}}{A_{15^{\circ}}} = 2.29$. The value of Q_{10} is variable and dependent on the interval of temperature considered. Beside the lack of constancy that the Q_{10} shows, this coefficient has another disadvantage: neither from its variations nor from its absolute values may we conclude anything relative to the type of process we study.

We may also handle these data in a graph showing the relation of CO_2 excretion

(ordinates) vs. temperature (abscissa), and the curve so obtained can be described by an empirical equation, with two or more constants. But one may question also if such an equation, which can always be written, will necessarily mean something; if the constants with the values there ascribed will represent some definite steps in the process or some characteristics of the system. In fact, in most of the equations belonging to this type, the constants are merely numerical factors having no real significance whatsoever. Such a case is found for instance in the formula given by Bělehrádek (1926): $y = \frac{a}{x^b}$ or $\log y = \log a - b \log x$, where y is the time, x the temperature *centigrade* and a and b are constants. This is the well known formula of Harcourt and Esson (1895) save that for no conceivable reason the absolute temperature has been replaced by the centigrade temperature. Such an equation enables one to fit with some exactness any series of observations where no abrupt change occurs, provided they do not go over a too great range of temperature; it furthermore allows the author in this case to assign to the constants such vague attributions as "viscosity of the protoplasm." For a review of reasons for the inapplicability of such formulæ the reader is referred to Crozier and Stier (1926-27). In résumé, such equations describe a curve but do not depict the process schematised by the curve or the mechanism underlying it. What we are interested in is a description which will throw some light on the kinetics of the process involved.

Numbers of vital activities may be considered as coupled systems of irreversible reactions. To such systems, the equation described by Arrhenius in 1889 may be applied, especially when we know that, although originally given as a purely empirical relation, it was found to submit to rational interpretation (*cf.* Tolman, 1927; Hinshelwood, 1927). This equation assumes that the velocity of an irreversible reaction is directly proportional to the exponential of $\frac{-\mu}{RT}$, where R is the gas constant, T the absolute temperature and μ the "energy of activation" per gram molecule of the "activated" substance. This expression, written habitually in the form

$$v_2 = v_1 e^{\frac{-\mu}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)}$$

with v_1 and v_2 the velocity constants (or proportional quantities) at the absolute temperatures T_1 and T_2 , gives μ directly, as from an adequate choice of coordinates and large scale units the value of μ is obtained by a single reading of the [20 inch] slide-rule (*cf.* Crozier, 1925-26, *e*).

This index is independent of the interval of temperature chosen, as well as of the absolute values of the velocities of the process studied. Despite what has been said by some writers, it is not affected by the aging of the organism, as a wide series of experiments in this laboratory has shown. If we consider μ as the activation energy of a catalyst, we may admit that processes activated in the same way will give the same μ ; and in the cases where the same catalyst occurs in different reactions

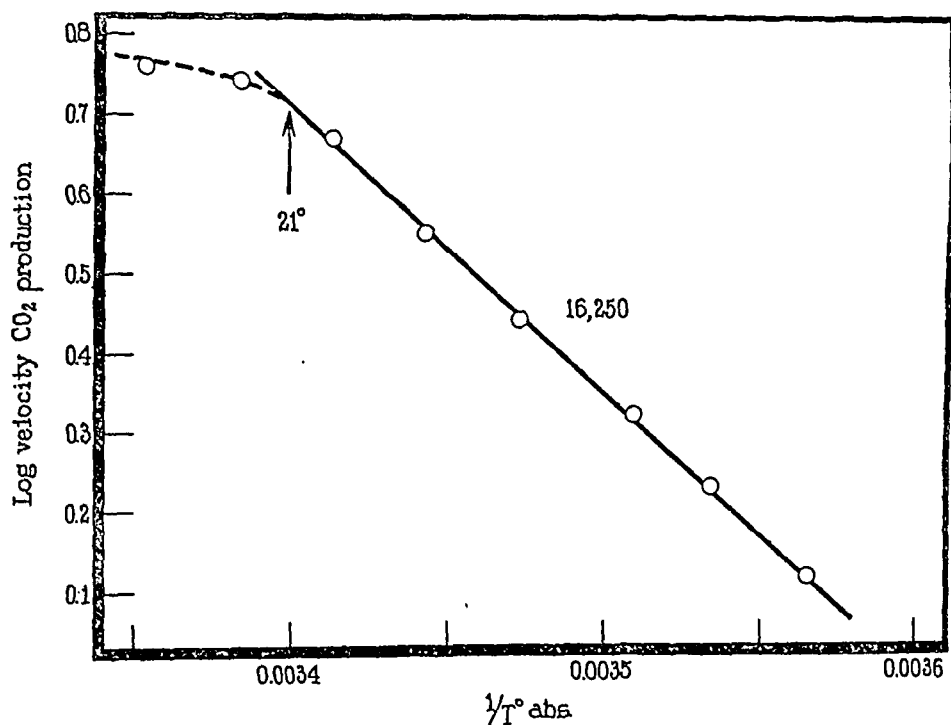


FIG. 3. CO₂ production by seedlings of *Vicia faba*.

we may expect to find the same value for μ . We may thus have an instrument which may give us indications as to the type of reaction controlling a series of coupled reactions. This method as used by Crozier (1924) has been developed with his collaborators in a series of studies which have plainly confirmed this viewpoint. It has furthermore the advantage of being easily applied when the observations have been made with due care; then it constitutes a real criterion of the precision in experimentation and of constancy in the operating conditions.

The data from our observations are rather striking in their good agreement with the form of the Arrhenius equation; the departure from the best fitting line is at any point at most of the order of the probable error. Moreover the value obtained (Fig. 3), $\mu = 16,250$, is one more in the list of oxidative respiratory processes yielding a critical increment of about 16,200. Compare this for instance with the values found in the published data: for oxygen consumption of *Arbacia* eggs (Loeb and Wasteneys), oxygen utilization of *Tenebrio* pupæ (Krogh), oxygen consumption of goldfish and of toad (Krogh),

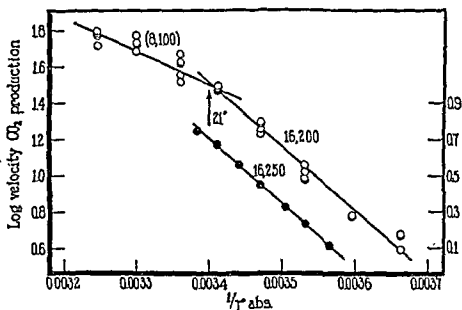


FIG. 4. CO_2 production by seedlings of *Pisum* (Kuyper) reduced to mg. per 100 gm. of seeds, per hour (open circles), and of *Vicia faba* reduced to mg. excreted by single seeds of 5.65 gm., per hour (solid circles).

where we get values from 16,100 to 16,800 (Crozier, 1924). Other instances since secured and collected have brought out this very constant value of μ for a large number of phenomena of that order. If the data of Kuyper are treated in the same way (Fig. 4) we find that they too give a value of μ around 16,200 for the lower range of temperature (up to 20°).⁶ Above that temperature we see a new level

⁶ The small discrepancy between this value and the one (16,500) published by Crozier (1924-25, c) is due to the fact that in the latter case, sets of data relative to each of the first 2 hours were plotted separately. For the present graph, on the contrary, all the data obtainable were plotted and the value of μ corresponds to the slope of the best fitting line for the whole.

being established with a different value of μ , just as is indicated in our case. It must be noted that in Kuyper's as well as in our data the critical temperature, determined by the intersection of the two best fitting lines, gives practically the same point on the thermometric scale, namely 21°C. A much larger departure between these values would not be disconcerting if we consider the heterogeneity of origin and technique in these different series of data. Their fair agreement points out once more that the values are not due to a mere chance distribution but on the contrary to a basic identity of the processes. We hold this very striking similarity of the values of μ as being highly significant for the theory of specific critical increments.

GEOTROPISM.

Reaction Time as a Function of Temperature.—It has been pointed out previously that the running of parallel experiments at different temperatures—these being distributed over the thermometric scale, in the range compatible with normal reactions—may give clues to the processes involved or to their kinetics, if the data are treated by the method described in the foregoing pages. We have seen that such a treatment of the mere numerical results obtained for the CO₂ excretion of *Vicia faba* has led us to obtain for the "temperature characteristic" (critical thermal increment)⁷ μ , the value of 16,250, which is in perfect accord with the normal value for respiratory oxidative processes.

In the same way, can we find some other indications which may lead to a view of the mechanism of geotropic curvature? Since a very long time, different authors who have treated this question have considered that two numerical time relations could be determined for such tropistic reactions. The first is the *presentation time*; the second, the *reaction time*. Text-books give generally the following definitions of these two terms (*cf.* for instance Benecke-Jost: *Pflanzenphysiologie*, 1923, ii, 263): *Presentation time* is the shortest time of exposure to the gravitational vector which induces a definite reaction detectable with the naked eye; *Reaction time* is the interval of time elapsing be-

⁷ The term "temperature characteristic" was introduced by Crozier (1924-25, b) to designate μ without theoretical implications, but this quantity has the dimensions of a critical thermal increment (calories per degree per gram molecule).

tween the moment when the stimulus is applied and the first reaction perceptible to the naked eye. It is apparent from these definitions that these time relations are susceptible to determination only in an approximate manner. But in any case, as we shall see, it is possible to obtain, under standard given conditions, results easily duplicated. In fact, the time of the first reaction observable depends naturally on the magnification of the picture of the organ considered. But one can take as a good indication the much better determined moment where the symmetry of the tip of the root, for instance, ceases and gives place to asymmetry. Furthermore, it should be noted also that these two factors seem to be correlated by Tröndle's rule in a definite and practically constant way for any given material. This rule states that if we vary the intensity of the gravitational vector (or its equivalent and easier handled centrifugal vector) we have a constant relation of hyperbolic form between presentation and reaction times. This is one of the reasons why we can consider presentation time as well as reaction time and even assume that the mechanisms involved in both relations may be very closely similar if not identical at *constant temperature*.

Let us note here also, as a general remark about all geotropic and phototropic experiments with plants, that what we are studying while investigating the reaction of the organism is always a lag period. In fact the chief effect we are following is the mechanism of bending and not directly the first part of the reaction resulting as an effect of a stimulating agency. We can perfectly well conceive that the organism considered in any field of excitation is instantaneously influenced by that field and that a certain "reaction" is started. That this "reaction" has to go on for a certain length of time is indicated by the lag period for the appearance of any external visible sign (*e.g.* a curvature) of "reaction." This curvature may be started only when the "reaction" has persisted for a given length of time or only when certain products determined by the "reaction" have been accumulated up to a certain level. And what we consider then habitually as a measure of geotropic reaction may be an expression of but one step in a chain of coupled reactions, but surely not the first one. This may also be why we can get for geotropic and for phototropic presentation times the same values for μ (*cf.* Crozier, 1924): that value of μ corresponding to

the initiation of the cellular work of bending an organ previously straight. To investigate the primary effects of light and of gravity as means of excitation, in relation to temperature, it would be necessary to introduce an additional variable (such as *intensity*). In the material studied by Castle (1927-28, 1928-29) for example, the temperature characteristics for velocity of elongation (growth) are not the same in different individuals; but at constant temperature the latent period of photic response (a growth elongation) is remarkably constant among a population of individuals.

The number of publications where the relation of presentation or reaction times to temperature has been considered is relatively small. The first data, given by Czapek (1895) and Bach (1907), are of a rather crude nature and seem to have been determined by a method which was unable to yield any better results. The manner of ascertaining the actual duration is open to severe criticism.

In 1912 Maillefer published a series of measurements which are remarkable for the careful determination of the reaction time in *Avena*. Their analysis will be given later. (This paper seems to have been quite overlooked by several authors.) The work of Rutgers (1912) was also executed very precisely; it is concerned mainly with geotropic presentation time in *Avena* and gives the best set of data existing for that quantity. These two papers can be used with confidence as the figures can be duplicated and have all possible reliability. The work of von Paál (1915) and of Tröndle (1915) on the effect of temperature on the geotropic reaction time is not usable, the control of temperature for instance being absolutely too vague. In von Paál's work, for instance, the temperatures are given under the form 22° to 23°, or 12° to 13°, and although experiments have been made at different temperatures his data can not be easily used. In Tröndle's work, the same lack of precision in the control of temperature prevails, and furthermore most of the experiments were performed in the range 18.5° to 25°C. which is too small to give precise indications and is in part *above* the critical temperature.

We are thus reduced to the two sets of data given by Maillefer and by Rutgers. For that reason also, we have tried to determine for the root of *Vicia faba* a series of reaction times which could be compared with the published tables.

The method was a standard one, used by Maillefer and by Rutgers, with the slight modification that the number of plants used was small for each set of experiments at any given temperature, but that the same sets were done several times under the same conditions. The figures obtained in successive runs agree so well one with another as to enable us to use them all together in one table. The variability in response described by von Paál, which in his account was said to become smaller and smaller as the experiment was performed at a temperature closer to the "optimum" temperature for the geotropic reaction, was not found at all in our series. It should be noted, also, that this

TABLE V.

Values of the Geotropic Reaction Time (R.T.) of Vicia faba Roots at Different Temperatures. Data from Czapek (1895), Bach (1907) and from the Present Experiments.

<i>t</i>	R. T.	Czapek's data	Bach's data	<i>t</i>	R. T.	Czapek's data	Bach's data
°C.	min.	min.	min.	°C.	min.	min.	min.
5	360*	360		17			115.4
7.5	190			20	57	80	97.9
10	150	120		22.5	51		
12	130			25	51	80	64.8
14			122.8	30		70	48.2
15	100	80					

* This R.T. may be too long; we have only three series of experiments for this temperature.

was not true in the experiments of Maillefer or of Rutgers either. The variability to which Maillefer refers is probably related to a lack of homogeneity in the strain of seeds used.

From this point of view, the material used in these experiments was remarkably constant in its reaction. All the plants used were always chosen to be as similar as possible one to another. The same thing has been found also in series of experiments where the reaction of the *stem* was considered. Table V shows in brief the mean durations of reaction time found for the different experiments.

In the third and fourth columns we have included the tables of Czapek and Bach, as they were the only other figures obtainable for

the same organism. Table VI contains the condensed tables of figures due to Maillefer and to Rutgers (experiments on *Avena*).

Maillefer does not seem to draw any conclusions from his observations, except as to the form of an equation describing the geotropic curvature, which he shows to be a parabola at least for the 1st hour of reaction. On the other hand Rutgers concludes that there is a non-direct relationship between reaction time and temperature, at least for the range 10° to 35°. He calculates Q_{10} and shows it to vary between 0.0064 (for high temperatures) and 6.8 (for the lower part of the thermometric range compatible with geotropic reaction). Be-

TABLE VI.

Values of Geotropic Reaction Time (R.T.) (Maillefer's Data) and of Geotropic Presentation Time (P.T.) (Rutgers' Data) for Avena sativa, at Different Temperatures.

<i>t</i>	R. T. (Maillefer)	<i>t</i>	R. T. (Maillefer)	<i>t</i>	P. T. (Rutgers)
°C.	min.	°C.	min.	°C.	min.
15	72.5	22	37.5	0	72
16	61.2	23	35.8	5	16
17	58.3	24	30.2	10	10.66
18	51.3	25	35.8	15	6
19	46.6	26	36.2	20	4.33
20	41.7	27	30.0	25	2.33
21	40.3			30	3.50

tween 5° and 35° he finds a $Q_{10} = 2.6$. He is thus in disagreement with Bach but not with Czapek.

All these conclusions seem to be very different one from another, even often in opposition one with another. Still, taking the data as they are published, it may nevertheless be possible to use them in certain ways. Let us apply to them the same treatment used for the data in the first part of this paper. We find (Figs. 5 and 6) the very striking result that the figures given by the different authors agree very well one with another as to the value of μ . For the different sets we find the following values: Maillefer, 16,200; Rutgers, 16,200; for my personal data, 16,110. All these figures obviously are within the normal range of variations that we may expect for such a value of μ by this

method of computation. It should be noted also that the first departures from the best fitting lines occur practically always at the same temperature: 21.0° , temperature that we found also to be a critical temperature for respiration in this form. We may note furthermore that 5° seems to be a second critical point, although not so well marked; but one must not forget that our observations at that temperature are few. These findings point to the same conclusion: the

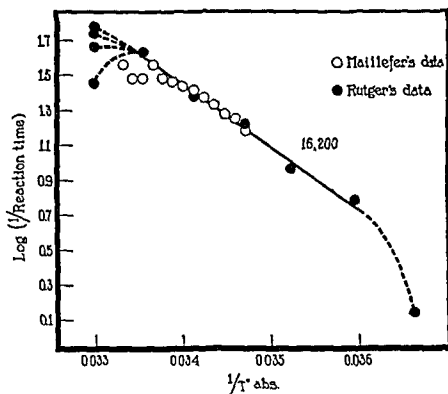


FIG. 5. The velocities of activation processes occupying the "presentation time" (Rutgers' data) and the "reaction time" (Maillefer's data) for geotropic response in *Avena*. The line corresponding to $\mu = 16,200$ fits both series with good agreement.

geotropic reaction in the coleoptile of *Avena* or in the root of seedlings of *Vicia faba* gives the same critical increment, 16,200, for reaction or for presentation times. This increment is known to occur in relation to a very large number of processes of respiratory oxidation and in our material also (*cf.* Crozier, 1924). It is then fair to conclude that in the processes underlying the geotropic curvature, a respiratory oxidative effect seems to be the master reaction determining the system of coupled reactions involved.

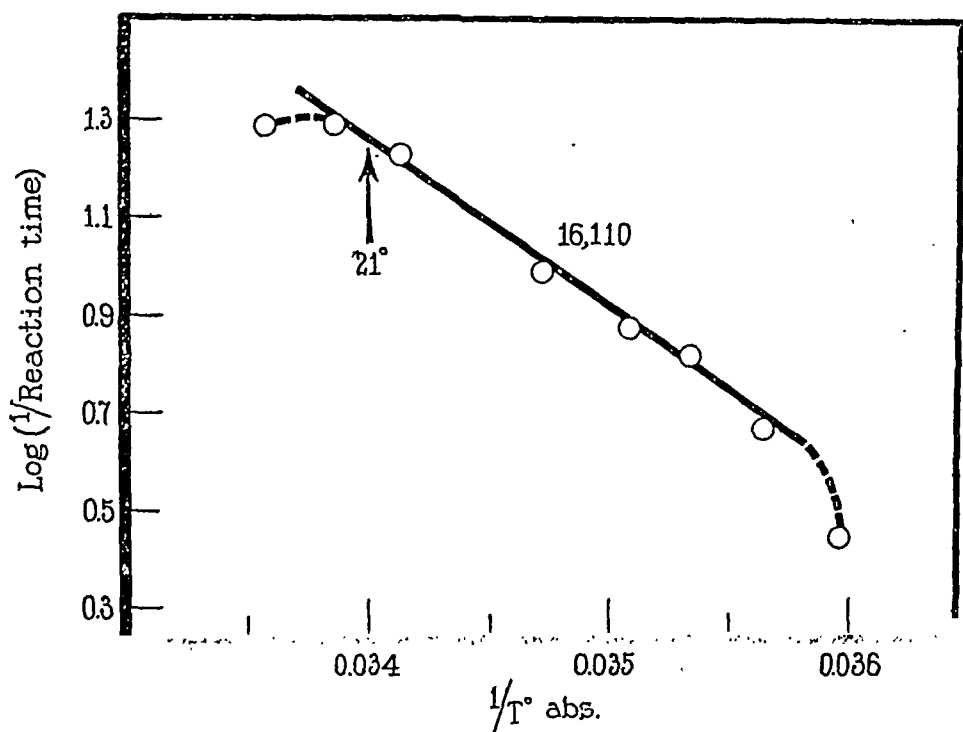


FIG. 6. The velocities of the activation process occupying the "reaction time" for geotropic response in roots of seedlings of *Vicia faba*.

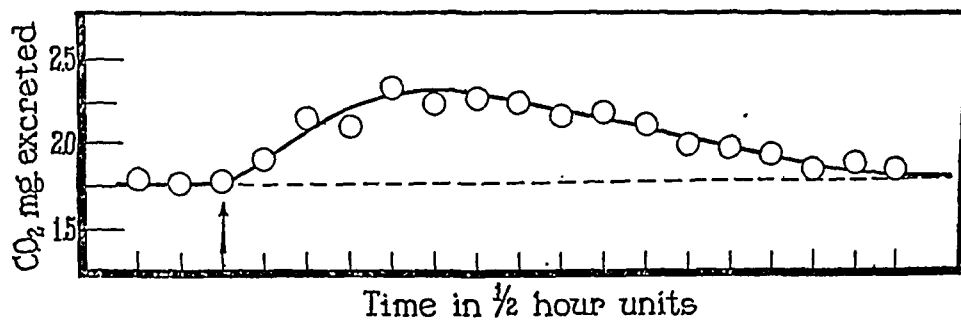


FIG. 7. Curve showing the increased CO_2 excretion when a seedling of *Vicia faba* is geotropically excited. The normal resting excretion is practically at a constant level; the arrow indicates the moment when the plant is turned through 90° and thus geotropically excited.

What this oxidative process is, is yet unknown; further experiments are necessary to ascertain the phases of that reaction; but that such a reaction is occurring, is within the range of certitude of our method of analysis.

An important fact which corroborates this view is the increased respiratory rate that one gets when a plant which has been treated so as to exclude in all prehandling, at any moment, the introduction of the gravitational vector, is subjected to this factor. Such a plant has for the resting rate of excretion of CO_2 a certain constant value even for long periods; if at any moment we rotate the respiration chamber through 90° , all the other factors being kept constant, we find that the rate of respiration increases up to a certain point, and later decreases and comes back after a certain time to its normal resting value. Such a curve, the typical form of which is retained with modifications of scale and of shape at each constant temperature, is given in Fig. 7. This gives a means of investigating the *amount* of geotropic response. The relation between this type of accelerated excretion consequent upon geotropic excitation and temperature will be found in the second part of this work. That such a type of change occurs is perfectly consistent with the values found for μ , for the reaction or presentation times. This may in part also explain why most of the authors who have determined the rate of excretion of CO_2 by seedlings got variable results: they were determining a mixed rate, perfectly uncontrollable, of CO_2 excretion in plants placed in all the intermediate positions between a normal resting state and a highly geotropically excited state. It can be added also that the temperature may even not have been under effective control, in the cases where the bulb of the thermometer was placed in the mass of seeds. This shows the advantage of the method where one seedling alone is used for each experiment and all the factors are under control.

SUMMARY.

In this paper there are given the results of a study of the relation of respiration to temperature, in seedlings of *Vicia faba*, and of the onset of geotropic response. It is shown that with due care and very accurate control of temperature constancy, one can get constant ex-

cretion of CO_2 even over fairly long periods. The treatment of the experimental data shows that the Q_{10} ratio is of course a valueless "constant," as it is variable; but that the figure obtained for μ , the temperature characteristic (critical thermal increment), 16,250, is perfectly consistent with the values previously obtained for μ in respiratory oxidative processes in similar material. New data on the reaction time for the root of *Vicia faba* seedlings excited geotropically are given also. The study of the dependence of this time relation on temperature shows $\mu = 16,110$, agreeing quantitatively with the value deduced previously from the relevant data of earlier investigators (Crozier, 1924). This points to the importance of some respiratory oxidative process as the agency controlling the onset of geotropic curvature.

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THE PHOTOTROPIC EXCITATION OF LIMAX.

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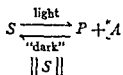
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I.

From measurements of the circus movements of *Limax* as governed by intensity of illumination it was deduced (Crozier and Cole; cf. Crozier, 1928) that over the range of intensities employed the mean amplitude of the orienting movement (degrees turned *per* centimeter of path) is directly proportional to the logarithm of the intensity. By a quite different method it was found (Wolf and Crozier, 1927-28) that with *Agriolimax*, which becomes much more quickly adapted by light, the velocity of photic adaptation is proportional to the logarithm of the intensity, as well as showing a logarithmic decrease in excitation with time of exposure to light of constant intensity.

The method employed with *Agriolimax* consisted in having the slug creep upon a vertical plate with the constant orienting effect of gravity continuously opposed by the action of a horizontal beam of light. The effect of the photic excitation is then to induce locomotion not straight upward, as in darkness, but at an angle β to the vertical away from the light. The course of light adaptation and of dark adaptation as measured by the changes in the magnitude of this angle can be fully formulated (Crozier and Wolf, 1927-28; 1928-29) on the assumption that the excitation is at any moment proportional to the rate of photolysis of sensitive material S , undergoing a first order decomposition by light and reconstituted by a "dark" reaction which is second order and with positive autocatalysis. With ordinary intensities of illumination, the course of the decomposition of S in the system



where $||S||$ indicates catalysis by S , is accordingly given by

$$-\frac{dS_t}{dt} = K_1IS_t - (K_2 + K_3S_t)(S_\infty - S_t)^2,$$

where S_∞ = the maximum amount of S which may be present at complete dark adaptation. Since K_1 is much greater than the other velocity constants (that is, light adaptation is here relatively much swifter than the reverse process of dark adaptation) the velocity of the light adaptation, under conditions imposed by threshold relations which must be satisfied in order to overcome the geotropic effect and thus to obtain any photic orientation, will appear very nearly proportional to $\log I$, as already found (Wolf and Crozier, 1927-28). In the case of a similar organism which becomes only very slowly adapted by the light it may be supposed that the rate of photolysis of S is actually very slow, in terms of the total amount of change possible, or that sensitive material is in some way supplied by an independent mechanism. Other possibilities may be appealed to, as related to the absorption of light in the sensitive cells, but in any case we might still expect that excitation would be nearly proportional to $\log I$ over such intermediate ranges of intensity as alone are open to test (*cf.* Hecht, 1919-20; 1922-23).

We have examined the behavior of *Limax* creeping upon a vertical plate with lateral illumination, to contrast its responses with those of *Agriolimax*, because its rate of light adaptation is much slower than with the latter form. From Yagi's (1927-28) experiments with *Dixiippus* we already have evidence of this kind for the proportionality of photic excitation and $\log I$ in a form which adapts only slowly to light. We desire to see, however, if the conclusion regarding *Limax* as based on the analysis of circus movements could be confirmed by this independent method.

II.

As a matter of convenience it has been usual to deal with fields of excitation which are geometrically simple. For the development of the method of tropistic analysis, however, it is required to examine more complex cases (*cf.* Crozier and Stier, 1928-29; Buder, 1917-19; Mitchell and Crozier, 1927-28). This is particularly necessary when

considering the compounding of tropistic effects (Crozier and Pincus, 1926-27; Crozier and Stier, 1927-28; Wolf and Crozier, 1927-28). It

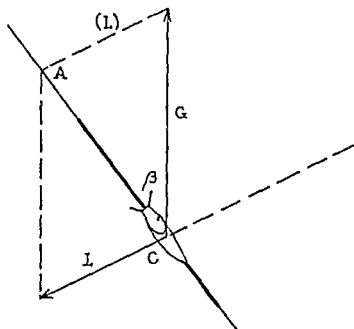


FIG. 1. A slug (*Limax*), previously dark-adapted, is creeping on a vertical plate of ground glass which has been moistened. It is illuminated at an angle from one side by light from a small condensed filament lamp. To avoid effects of the observer's breathing currents, the path of the slug is observed from the other side of the plate. At the point *C* the slug is under the influence of an orienting field containing two significant vectors: a gravitational vector *G*, which is assumed constant; and a photic vector *L*, of magnitude assumed proportional to $\log I$. *I* is measured at *C*, with a photometer giving illumination in foot candles. The direction of progression is given by the heavy line *b*. Then if the assumptions are valid

$$L = K \log I = \left(\frac{G}{\sin A} \right) \times (\sin \beta)$$

or,

$$\frac{K' \sin \beta}{\sin A} = \log I \quad (2)$$

The plot in Fig. 2 shows that the equation is satisfactorily obeyed.

becomes desirable to formulate orienting movements when the competing excitations do not act at right angles or directly in opposition (Crozier, 1926-28). An elementary instance is given by the following

series of experiments, in which the difference from those cited in the previous section lies in the angular inclination of the photic and the gravitational vectors.

Numbers of *Limax* have been at various times thoroughly examined for geotropic orientation, with the result that in general it is difficult to obtain clear-cut connections between the slope of the surface and the amount of upward orientation (or of downward orientation during geopositive phases). This is largely due to the fact that geotropic orientation is evidenced only during active progression, and

TABLE I.

Observed Values of the Angle of Orientation Away from Vertical (β), at Various Magnitudes of Intensity of Illumination (I) and of the Angle of Incidence (A) of the Light.

I	A	β
<i>foot candles</i>	<i>degrees</i>	<i>degrees</i>
2.6	98.8	41.4 \pm 3.4
2.5	101.6	36.8 \pm 4.9
2.3	96.4	37.8 \pm 5.4
1.53	87.6	33.0 \pm 4.5
1.25	90.0	33.1 \pm 4.0
0.96	84.6	27.9 \pm 4.1
0.77	90.0	25.1 \pm 3.6
0.57	82.9	23.5 \pm 3.7
0.52	83.5	22.0 \pm 4.6
0.48	86.1	18.8 \pm 3.1

is effected by the speed of progression, within certain limits (*cf.* Crozier, 1928). We have found it better, therefore, for the present purpose to utilize groups of young *Limax* hatched from egg masses in the Laboratory and raised to a length of approximately 1 cm., being fed on decaying wood, and apple and the like. Such individuals are occasionally indifferent to light (*cf.* Crozier and Libby, 1924-25; Crozier and Federighi, 1924-25). Provisional testings are therefore required for the selection of sensitive individuals. So far as possible such individuals were used in a series of tests at several intensities. The animals were handled as in the experiments described by Wolf (1926-27) and Wolf and Crozier (1927-28; 1928-29).

The conditions in the tests are given diagrammatically in Fig. 1. The measured angles are β and A ; O is the mid-point of the oriented trail on the vertical plate. Then, G being assumed constant, and if L be proportional to $\log I$, the condition which should be obeyed is:

$$\log I = \frac{K \sin \beta}{\sin A} \quad (1)$$

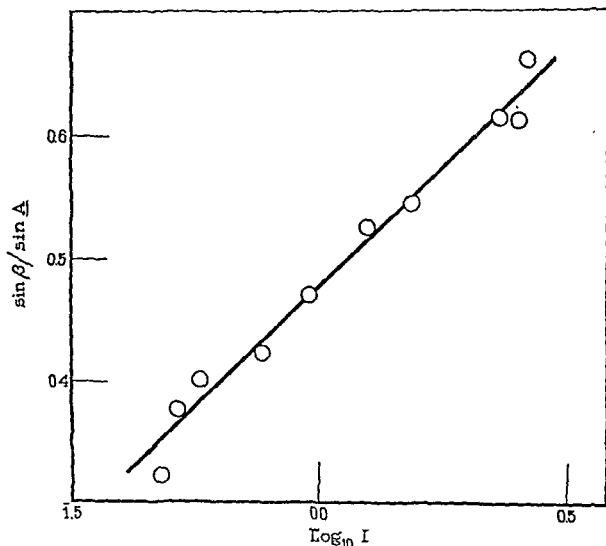


FIG. 2. $\log I$ plotted against $\sin \beta / \sin A$ (cf. Fig. 1) shows a rectilinear relationship. Data in Table I. The residual variation (Table I) is approximately constant throughout (*ca.* $\pm 4.0^\circ$).

The data are given in Table I, and are plotted in Fig. 2. The agreement must be considered very good. The deviation at the lowest intensity is fully covered by the probable error, and in fact a positive error of 1.2° of arc in the mean value of β (easily possible in view of

the inevitable crudity of the measurements) would bring this point back on the line fitted to the observations.

SUMMARY.

The photic orientation of *Limax* creeping geotropically upon a vertical plate is such that the phototropic vector determining the angular deflection β from the vertical path is proportional to $\log I$. This is proved by the fact that with horizontal illumination $\tan \beta$ is directly proportional to $\log I$; with non-horizontal light rays from a small source the ratio $\frac{\sin \beta}{\sin A}$ is directly proportional to $\log I$ (where A = the angle between light rays and the path of orientation), the vector diagram of the field of excitation being in this case not a right-angled triangle.

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GEOTROPIC ORIENTATION IN ARTHROPODS.

II. TETRAOPES.

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I.

The geotropism of certain arthropods offers opportunity for the evaluation of descriptive equations derived from the behavior of forms already studied. If these equations, for example those for the geotropic orientation of slugs (Wolf, 1926-27) and of tent caterpillars (Crozier and Stier, 1927-28), are based upon a correct understanding of the mechanisms which essentially limit orientation, then a geotropic organism of different morphology, or with different method of progression, should be expected to obey somewhat different rules, in correlation with any obvious difference in the way in which the weight of the body can be supported upon an inclined plane. This turns out to be the case. There is thus provided a proof that the quantitative formulations previously discussed are not mathematical accidents in some way due to the averaging of observations. This kind of test has been made with the negative geotropic orientation of the milkweed beetle *Tetraopes tetraophthalmus*. The case of the fiddler crab *Uca* has been examined elsewhere (Kropp and Crozier, 1928-29), and the behavior of several other forms has also been studied in detail; the latter instances will be discussed in another place.

II.

Tetraopes was collected on milkweed plants and kept in the laboratory in terraria upon transplanted milkweeds. The animals are hardy, and submit in a satisfactory way to a variety of manipulative operations. Their geotropism is normally precise and negative. The oriented upward progress is however periodically interrupted by brief intervals of cessation of creeping, even upon a vertical surface, which

may be accompanied by more or less vague turning movements, and at times by transient but definite *positive* orientation. Here, as in other instances, there accordingly appears an important correlation between continuity of progression and precision of geotropic orientation. Moreover, the equilibrium angle of orientation, θ , also varies, within certain limits, and with fairly definite rhythm, as already mentioned for caterpillars (Crozier and Stier, 1927-28). It is notable that in the cases of geotropism with which these experiments have been concerned the assumption of a significant alignment of the axis of the organism upon the plane (as defined by the angle θ) is apparent only *during progression*. A similar relationship between orientation and creeping is evident in experiments upon a turntable. It may be taken to mean that the central nervous condition attending the rhythmic use of the appendages (or, with caterpillars, perhaps the body musculature also) in creeping permits the effect of tensions applied to the muscles to exert an influence upon orientation. This may be due, however, to the fact that *change* (increase) of tension is the effective stimulus for the tension receptors, as is true in vertebrates. Interesting questions arising from such considerations, and in connection with the intermittent failure to maintain orientation, we must put aside for the present with this bare notice of their realization.

The geotropism of *Tetraopes* is very strikingly affected as a result of mechanical stimulation such as that due to handling. In this respect there is a certain parallelism with the reversals of phototropism seen in various animals as a result of mechanical excitation. The method used to obtain geotropic trails avoided any such disturbance. As the beetle approached the upper edge of the sheet of white paper on which it was creeping upward, a small piece of paper held at one corner by the experimenter was placed in its path; when the beetle had mounted it, the paper was moved to the lower edge of the recording sheet, where, after suitable rotation of the carrier, the animal was allowed to creep back again upon the record sheet. The rotation was necessary to enable the new trail to be initiated with a path almost horizontal in direction, from which upward orientation followed. The absence of any special effect due to handling is attested by the uniformity of the results as gotten by each of the two observers.

Trails were also obtained by the method earlier described for tent

caterpillars, in which a platform 3 meters long permitted very extensive creeping. In this way it was possible to study spontaneous changes of the angle θ , and the periodic halts and other phenomena associated with such shifts.

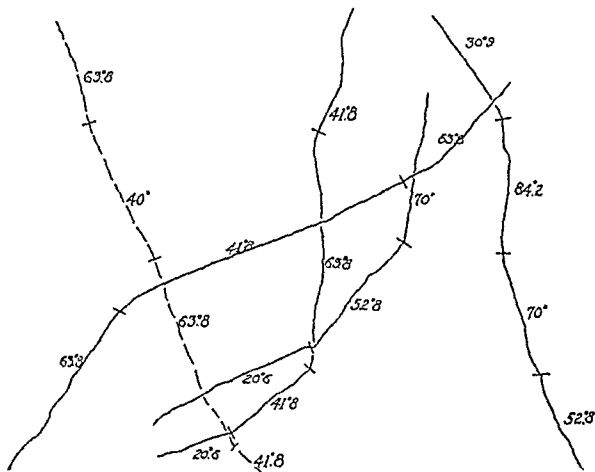


FIG. 1. Tracings of trails, selected at random, to show dependence of θ , the angle of upward orientation, upon the indicated tilt (α) of the plane upon which *Tetraopes* is creeping. Individuals temporarily asymmetric in response, i.e., tending persistently to turn to right or to left, were purposely chosen for these tests in which α was altered (increased or decreased) without touching the beetles; for this reason the magnitudes of α are lower than those obtained as general averages (cf. Table I, Table II, and text).

It is desirable to realize that the dependence of the angle θ upon the inclination α can be demonstrated by a technic which avoids any possible consequence of immediate handling of the animals, and which is largely unaffected by spontaneous fluctuations in geotropic sensitivity. (This particular procedure, also employed by Crozier and Pincus (1927-28) in experiments with rodents, not yet described, has

the advantage that it permits a subsequent study of the latent period for geotropic response.) It consists in tilting the creeping plane at various angles, during progression of the animal upon it. The method has with some forms a certain restriction. If the inclination be *decreased*, the assumption of a new orientation may be delayed and vague, in agreement with the conception that in the field delimited by $+\theta$ and $-\theta$ orientation is not constrained. *Tetraopes*' behavior, however, allows this restriction to be circumvented. By choosing pronouncedly "asymmetric" individuals such as (for the time being) turn persistently to right or to left, the geotropic orientation is forced to work against a "brake" and thus to be expressed more delicately. Illustrative trails are copied in Fig. 1. The mean values of θ from such experiments, given in Table II, (Fig. 4) are naturally lower than with symmetrical average sensitivity (Fig. 3).

In one respect the experimental conditions differed from those required with the caterpillars. Like certain other beetles, *Tetraopes* is geotropically active consistently only in light. In darkness, creeping is usually absent, or aimless. It was shown by Moore and Cole (1920-21) that *Popillia japonica* creeps vertically (on a vertical surface) more rapidly as the illumination is increased, quite apart from any *phototropic* influence. But the photokinetic effect does not influence the *angle* of orientation on an inclined surface, in *Tetraopes*. Nor does the position of the source of light, in spite of the positive phototropism which is pronounced under other conditions (Crozier and Mangelsdorf, 1923-24). Our experiments, however, were made in a dark room with light (and diffusing screen) directly above the creeping stage, of 40 m.c. intensity. With such illuminations, light may come either from below, ahead, or from one side of the animal without modifying θ . With light of much lower intensities it is apparently possible to compound positive phototropic and negative phototropic orientations upon inclined surfaces, but the very interesting additive and threshold relationships between vectorial phototropism, geotropism, and photokinesis we have not as yet been able to examine in detail. Occasionally, good creeping was had without high light intensity. One such instance was given by beetle 16. With $\alpha = 49^\circ$, trails under white light gave $\theta = 74.48^\circ$; under weak red light, $\theta = 74.60^\circ$. In subsequent experiments with *Pyrophorus* (Kropp, 1929) observations could be made in darkness.

The absence of effective directional action by light of higher intensities in these experiments has one fortunate consequence. The use of rough white paper as a creeping surface did not introduce errors due to orientation by reflected light. This was checked by comparison with trails upon dull black cloth. It is nevertheless true that when in geotropic creeping on white paper the beetle closely approaches the boundary of a black surface upon which the paper rests, the trail suffers noticeable deflection. But it is only such contrast as between white and black areas which produces this temporary dis-

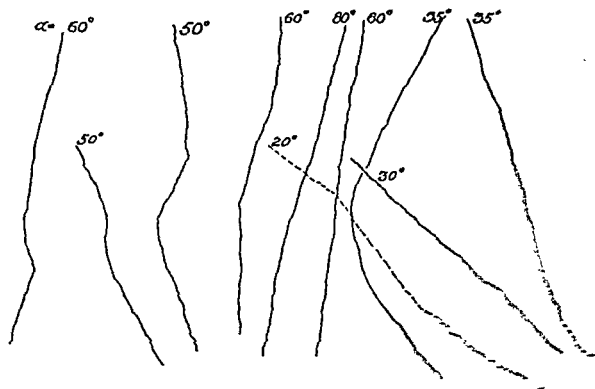


FIG. 2. Various trails, chosen at random, to illustrate the response of orientation of *Tetraopes* on inclined surfaces; see text.

more frequent. This was also studied with beetles enclosed in a large spherical flask immersed in a thermostat at controlled temperatures. The spasms of elytral opening occur at longer intervals at 24° , although

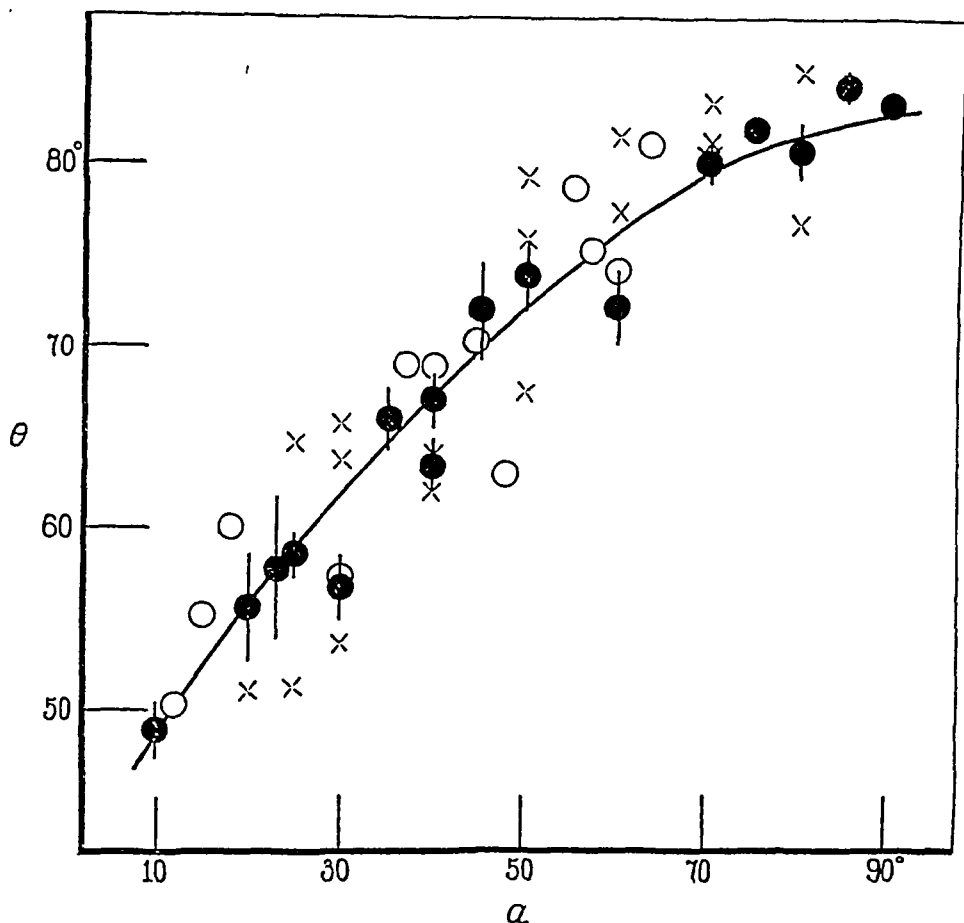


FIG. 3. Mean magnitudes of angle of orientation θ as function of the inclination of the surface α . Values which are averages from fewer than ten series of measurements are given as open circles, of ten or more as solid circles (with the probable error of the mean of the series' means indicated). There are added (crosses) mean estimates of θ from tests with a single individual (No. 15) used at a large number of inclinations. The curve is that of $\Delta \theta / \Delta \sin \alpha = \text{const.}$ (cf. Fig. 7).

even here an activity of this kind may *rarely* eventuate in flying departure from even a flat surface. To obtain creeping uninterrupted by these disturbances, the geotropism tests were made at temperatures between 23.5° and 25° .

No differences in orientation were observed which could be correlated with sex or size of individual, and the results from all have therefore been taken together. Between 10 and 20 trails were secured, at various angles of inclination of the supporting plane, with each of some 56 individuals. Not all of these were used at every inclination, some being chiefly employed for operative experiments.

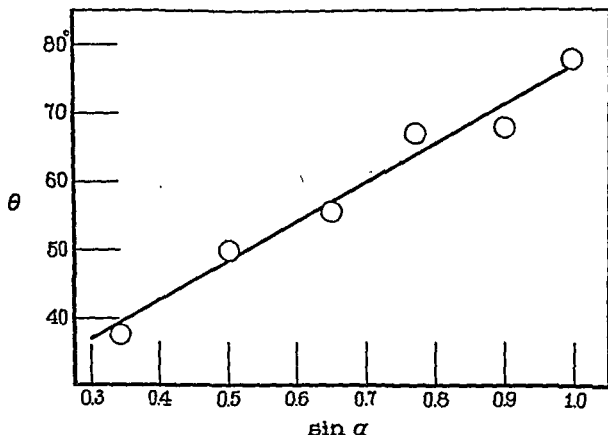


FIG. 4. Mean values of θ as a function of $\sin \alpha$, from trails with 7 markedly asymmetric individuals (cf. Fig. 1, and text). The values of θ (Table II) are lower than the averages at corresponding inclinations with normal (*i.e.* not asymmetric) individuals.

A total of approximately 3,400 trails form the basis of Fig. 3. The variability of θ , under these conditions, cannot be expected to show the sort of regularity discovered in other experiments with groups of selected individuals, and it is significant that the variation of the mean θ 's as tabulated cannot be reduced to any simple rule. The angles of orientation (θ) were measured upon the trails as accurately recorded with soft pencil during the progress of creeping. Illustrative details are given in Fig. 2. As with the caterpillars, there occur periods of

marked asymmetry of response. During such a period it is sometimes impossible for several hours to obtain a single trail with orientation to the right (or to the left, as the case may be) but all go to the opposite side no matter how the beetle is started creeping. Several

TABLE I.

Mean Angles (θ) of Upward Orientation of Tetraopes on a Plane Inclined at α° with the Horizontal. The Values of θ Are Not All of the Same Weight; Cf. Fig. 3.

α	θ	α	θ
<i>degrees</i>	<i>degrees</i>	<i>degrees</i>	<i>degrees</i>
10	48.89	45	71.99
12.7	50.21	46.5	63.13
15	55.25	49	74.60
18	60.02	50	73.99
20	58.04	55	78.78
23.3	62.15	57.1	75.69
25	58.96	60	72.2
28	62.92	63.3	81.17
30	61.64	70	80.17
35	66.04	75	81.95
37	68.63	80	80.77
40	63.50	85	84.35
44.3	70.3	90	83.3

TABLE II.

Mean Angles of Upward Orientation (θ) from Experiments in Which 7 Tetraopes Markedly Asymmetric in Response Were Allowed to Creep upon a Platform the Slope of Which (α) Was Subsequently Altered to the Value Noted (See Text, and Fig. 1).

α	20.6°	30.9°	41.8°	52.8°	63.8°	84.2°
θ	37.78°	50.00°	55.51°	67.08°	67.99°	77.95°

hours later, or next day, the orientation of this individual may be equally definite but to the other side. We have not attempted, in such cases, to apply the method of averaging used with the tent caterpillars, but have simply taken the values of θ , in view of the absence of any systematic divergence. Creeping is slower at higher values

of α (and of θ), but this was not studied systematically. In every case the beetles used showed a normal activity of the legs in creeping; it is *particularly* necessary to be sure that the tarsal hooks have not been torn off when the animal is removed from the milkweed, or during subsequent handling. Deficiencies of this kind, or injuries to the

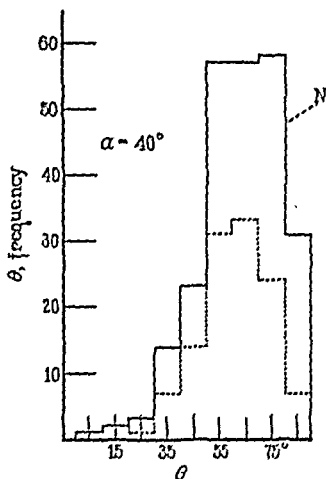


FIG. 5. Distributions of measurements of θ for all observations at $\alpha = 40^\circ$; for normal individuals (N) and for those with antennae removed.

legs, quickly reveal themselves in irregularities of orientation and in circling movements; the functional significance of the legs differs among the 3 pairs and all 6 legs are required for adequate progression, as proved by a variety of amputation tests.

III.

The average angles of upward orientation (θ) of *Tetraopes* are related to the tilt of the creeping surface as set out in Table I (Fig. 3). Each average is the mean of the averages of θ for each of a number of

individuals. The manner in which the separate readings of θ are distributed about the means is illustrated in Fig. 5. These distributions are essentially symmetrical, at inclinations below $\alpha = 60^\circ$. The proportionate scatter in such graphs is not fairly dealt with unless the size of the θ - classes is made less in proportion to $\sin \alpha$; this is due

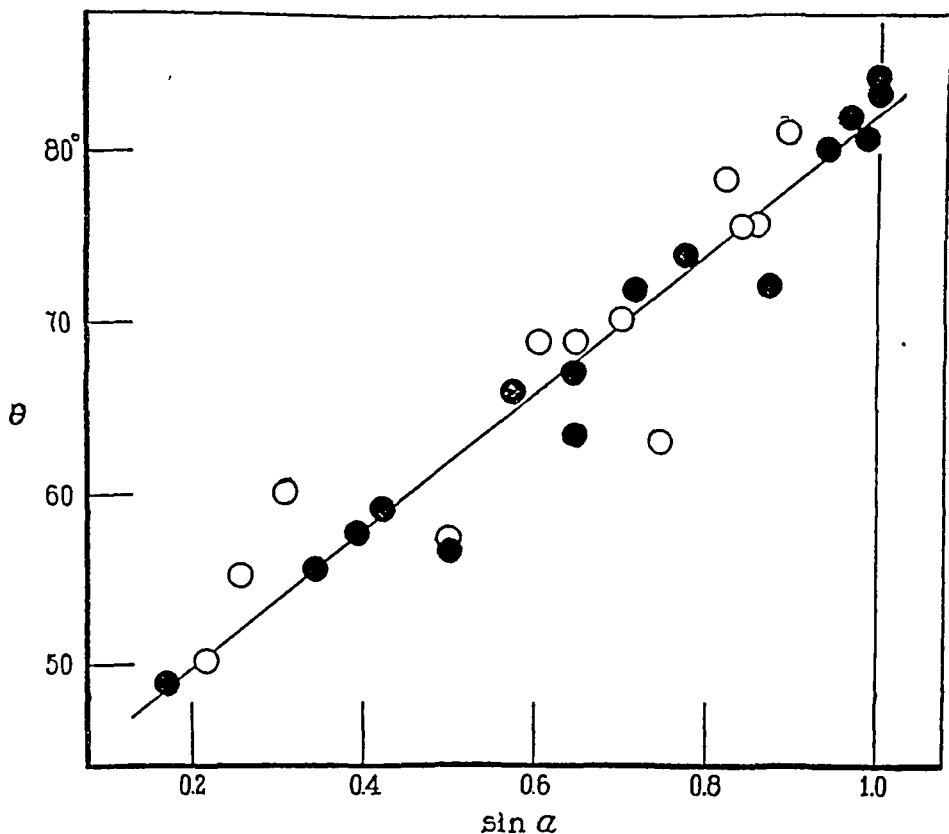


FIG. 6. The relation between θ and $\sin \alpha$ is practically linear (*cf.* Fig. 3); weighing of the points is as indicated in Fig. 3.

to the fact, brought out in earlier accounts (Crozier and Pincus, 1926-27, *a, b*), that the variability of θ is itself a function of $\sin \alpha$. Such a result provides an important test of the adequacy of the experimental procedures. At high inclinations the distribution of θ is necessarily somewhat skew, since the animal cannot very well give $\theta > 90^\circ$. The situation here as regards the distribution of θ 's is pre-

cisely that already described in our preceding paper (Crozier and Stier, 1927-28, b). The distribution of the mean values of θ obtained from the several individuals is also "normal." A sufficient method of weighing these means is to give the number of "runs" of observations concerned in each. In Fig. 3 the points based upon averages from fewer than 7 individuals are entered as open circlets.

Examination shows that in the case of *Tetraopes* the angle θ is linearly proportional, not to $\log \sin \alpha$ as in the earlier cases, but to $\sin \alpha$ directly. The lowest tilt at which consistent orientation is obtained is $\alpha = 15^\circ$; below this, positive and negative orientations fluctuate, or creeping is random. With some individuals the threshold is at 20° . With angles above $\alpha = 85^\circ$ creeping is slowed, and no increase in θ is to be detected. Between these limits the ratio $\Delta\theta/\Delta \sin \alpha$ is constant (Fig. 6).

IV.

Inspection of the creeping beetles at once suggests a possible origin for the difference between the descriptive formula for their orientation and that for the other forms thus far considered. The direct rather

than logarithmic relationship between θ and the sine of the inclination of the surface, whatever its mechanism, is incidentally a valuable commentary on the point previously made (Crozier and Pincus, 1926-27, a, b, c; Crozier, 1928; and Crozier and Stier, 1927-28) regarding the "Weber-Fechner Law." In *Tetraopes*, as with certain other beetles, the center of gravity is posterior to the region of attachment of the legs (Fig. 7). This must result in a turning moment applied to the legs, in addition to the straight downward pull which they support parallel to the inclined plane. The same effect is seen in elaterids; of these, several luminous Cuban species were studied be-

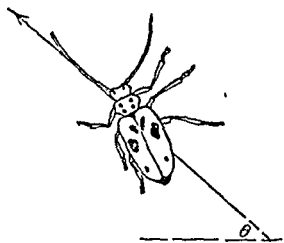


FIG. 7. The posture of *Tetraopes* upon an inclined surface. Owing to the mass of the abdomen, a twisting moment must be supported by the legs when $\theta < 90^\circ$. Diagrammatic.

cause it is possible to follow their paths in darkness. If the notion be correct that the gravitational pull supported by the legs during creeping supplies the gravitational stimulus, and also, because of a differential distribution upon the appendages of two sides, provides the limiting condition necessary to establish θ as a function of α , then the relationship should be experimentally controllable by modifying the

TABLE III.

Results of Experiments in Which the Distribution of the Weight, and the Total Load, Carried by Tetraopes during Upward Geotropic Creeping Was Varied.

1. A small mass of colophonium cement weighing 3.8 mg. attached to the posterior dorsal surface of the clytra.

α	θ	θ with wax attached
25°	61.1	68.4
55	80.3	84.6
75	82.0	81.93

2. A small mass of cement attached to the dorsal surface of the thorax.

α	θ	Attached mass	θ with wax attached
25°	61.1	3.8 mg.	57.6°
35	64.5	21.2 mg.	35.4 (90 per cent trails +)
	74.04	3.8	48.6 (30 per cent trails +)
55	80.3	3.8	63.4
75	82.0	3.8	81.2

3. The wing covers and wings removed.

$\alpha = 40^\circ$; mean θ for the individuals used in these tests = $67.1^\circ \pm 1.5^\circ$ before removal of wings; $\theta = 69.0^\circ \pm 2.0$ after removal.

(Removing the antennæ, also, has no detectable effect).

several components of this pull. Removal of the antennæ of *Tetraopes* has no effect on orientation (*cf.* Fig. 5). In land pulmonates (Crozier and Federighi, 1924-25; Cole, 1925-28; Crozier and Navez, 1928-29) and in young mammals (Crozier and Pincus, 1926-27, *a, b*; 1927-28; Pincus, 1926-27) control through effect on the tensions of muscles is quite clear; in certain other instances, according to a few tests made, for example, by Fraenkel (1927) with *Littorina*, and

(unpublished) by Mr. E. F. B. Fries with *Dendronotus*, the effect if any is dubious with these particular aquatic forms. The experiments with some forms are hampered by the nature of the creeping mechanism (*Dendronotus*); in the case of others it is first necessary to remove the possible influence of other sources of stimulation, and to make certain that the animal is moving with adequate speed (as has been found with *Littorina* creeping in air). In still other cases, among such pulmonates as *Limax*, for example, comparative measurements can only

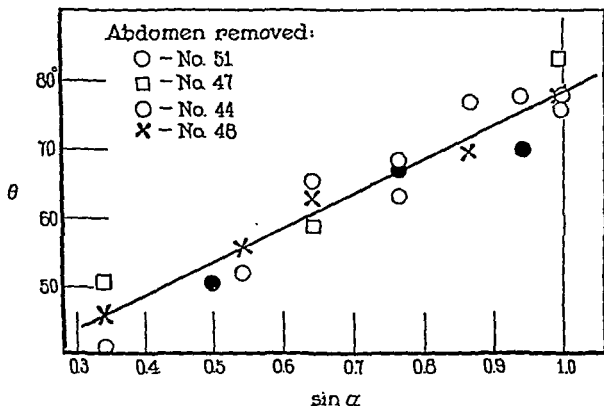


FIG. 8. Mean values of θ at various slopes (α) of the surface on which creeping takes place, for 4 individuals after amputation of the abdomen.

be obtained when the animals are creeping with comparable degrees of extension of the body, since the tonus or the degree of extension of the musculature must be supposed to influence the sensitivity of its proprioceptors to deforming pressures; this rather elementary kind of error (together with several others) has for example led Piéron (1928) distinctly astray, as our own numerous earlier experiments with *Limax maximus* suffice to demonstrate.

With *Tetraopes* it is a simple matter to perform experiments of the following kinds: 1, an additional mass (a small bit of colophonium

cement) is attached at the posterior margin of the elytra; 2, a similar addition is made to the head or at the anterior border of the thorax; 3, the wing covers and wings may be removed; 4, the whole abdomen may be cut away.

Experiments of type 1 should increase θ , slightly, at constant values of α , if α is not too large; in type 2, θ should be decreased; in 3, θ should be very little affected, but if anything decreased; in 4, it should be markedly decreased. The results obtained in a long series of trials agree with these predictions, in every particular. For most of the tests the inclination $\alpha = 40^\circ$ was chosen. The findings are summarized in Table III, which deals with the first 3 kinds of modification. The effect of adding a load anterior to the attachment of the legs being to decrease the effect of the turning moment about their lines of intersection, the fact that θ is decreased, despite the augmented total load, proves the importance of this factor.

The effect of removing the abdomen must be considered in greater detail. It is expected to do two things: first, to lessen the total downward pull; second, to remove the twisting moment on the legs. The operation is made in such a way that the legs are not directly injured, and by cutting around the chitin before cutting through the internal organs there is less extensive damage to the contents of the thorax. The cut surface is covered by a thin layer of vaseline, to prevent evaporation. Such preparations live for several days. After an interval for recovery they creep fairly well, and their orientation is definite. Mean values of θ from experiments with 4 individuals used at a number of inclinations are collected in Fig. 8; θ is consistently lower than with the intact animals (Fig. 6). In terms of the preceding suggestions, the lower values of θ resulting after amputation of the abdomen should be corrected by attachment of a substitute mass of wax. Thus in the case of *Tetraopes* No. 51:

$$\begin{aligned}\alpha &= 35^\circ \\ \theta &= 65.0^\circ \\ \theta, \text{abdomen removed,} &= 48.9^\circ \\ \theta, \text{abdomen removed} &= 75.6^\circ \\ &\text{but with load of} \\ &\text{wax (8.9 mg.) attached in its place,}\end{aligned}$$

If the turning moment due to the abdomen is responsible for the failure of an approximately logarithmic connection between θ and $\sin \alpha$, then

such beetles should in their orientation obey a formula different from that obtained for the normal *Tetraopes*. Experiments with several active preparations are summarized in Fig. 8. It is apparent that the relation of θ to $\sin \alpha$ is however again direct, as nearly as can be told, rather than logarithmic; the difference must arise in some other way.

These experiments give, then, an additional check upon the theory of geotropic orientation, in two particulars: (1) the different formu-

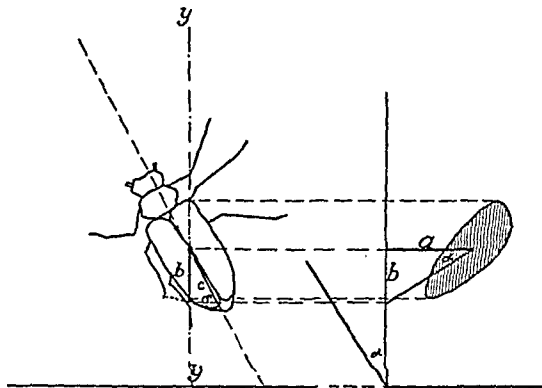


FIG. 9. Projection of a creeping *Tetraopes* upon an inclined surface (diagrammatic), and at the right a vertical section in the plane $y \dots y$. The line of gravitational pull passes downward from the center of gravity, and is assumed (as a limiting condition) to pierce the surface on the line connecting the tip of the abdomen, which is in contact with the surface, with the tip of the midmost position of the mesothoracic leg. See text.

lations required according to differences in structure, and (2) a test of the muscle tension theory when the gravitational pull can be, not merely increased as in former experiments, but also decreased as well. It remains to find a rationalization of the quantitative relations in the case of *Tetraopes*. This is done in the following way. Let it be considered that the sideward displacement of the body axis through the pull of the abdomen is for the moment ignored. Then the conditions

of progression may be diagrammed as in Fig. 7 and in Fig. 9, in which the outline of the beetle is projected normally upon the plane of creeping. But in fact the body is effectively supported at 3 points only, namely, by the legs on either side and by the posterior end of the abdomen (it is true that the mandibles are also, at each step, in brief contact with the substratum), and except at these points it is not in contact with the plane. A prime condition for stability, then, will be that the vertical line from the insect's center of gravity must pass through the plane at a point within the "base of support" defined on the downward side by the line drawn from the legs (taking the mesothoracic leg as representing the mean position) and the tip of the abdomen. A section in the vertical plane $y \dots y$ then discloses that $\tan \alpha = \frac{b}{a}$, where b is defined in Fig. 9. From the projection on the creeping plane,

$$b = \frac{x \sin (\pi - \delta)}{\sin (\pi - 90^\circ + \theta - \delta)}$$

therefore

$$\frac{a}{c} \tan \alpha = \frac{\sin (\pi - \delta)}{\sin (90^\circ + \theta - \delta)}$$

Since a and c , coordinates of the center of gravity, may be assumed constant, and likewise the structural angle δ ,

$$\begin{aligned} K \tan \alpha &= \frac{1}{\sin (90^\circ + \theta) \cos \delta - \sin \delta \cos (90^\circ + \theta)} \\ &= \frac{1}{K' \sin \theta + K'' \cos \theta} \end{aligned}$$

$$K_1 \cot \alpha = K_2 \sin \theta + \sqrt{1 - K_2^2} \cos \theta.$$

Values for the constants may be gotten by substituting pairs of values of α and θ . A plot of this relationship should show, if the interpretation be sound, a rectilinear decrease of $K_2 \sin \theta + \sqrt{1 - K_2^2} \cos \theta$ with $\cot \alpha$. The theory of this relationship assumes merely that during geotropic creeping *Tetraopes* turns upward (or downward, in case of positive geotropism) until tension on the legs such as would (unap-

posed) lead to tumbling off the surface is relieved by the attainment of a mechanically stable posture on the inclined plane. It will be brought out subsequently that this simple assumption leads to very interesting results when used to interpret the geotropic orientations of certain other forms (e.g., *Uca*, cf. Kropp and Crozier, 1928-29) and of positively geotropic creeping. (Qualitatively, the fact was demonstrated with *Tetraopes* that similar changes of θ with α appear when the animal creeps on the under surface of a board (cf. also Wolf, 1926-27).)

With *Tetraopes* however, an additional feature must be considered. The downward slewing of the abdomen (Fig. 7) causes θ to be less, at a given magnitude of α , than if the axis of the body were rigidly perpendicular to the effective "axis" of the legs. This prevents obtaining a good fit of the observed θ 's by means of the foregoing equation at low values of α . It is as if the animal responded to the requirement of stability not merely by the upward orientation but also by turning the abdomen to one side, thus broadening on that side the base of support. A shifting of the center of gravity, by attaching bits of wax, should in the case of a backward shift cause θ to increase somewhat, as is obvious from Fig. 7, and this in spite of the inclination of the beetle's axis to the plane. A forward shift should cause the limiting value of θ to decrease, for similar reasons, since the base of support broadens up to the points of application of the legs to the substratum. These effects have already been recorded. A further difference appears at quite high values of α , since then the support at the tip of the abdomen becomes inappreciable. The simplicity of the satisfactorily direct proportionality between θ and $\sin \alpha$ (Fig. 6) therefore appears to cloak a variety of complications. In the extreme case, when the abdomen is removed, the situation is somewhat altered. We then

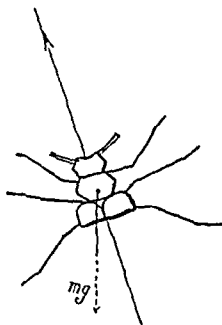


FIG. 10. Diagram of the conditions of oriented progression in *Tetraopes* with abdomen amputated.

conceive the base of support (at low inclinations of the surface) to be limited by a line connecting the points of opposition of the legs on the "down" side. At higher values of α , this condition cannot be met, and orientation must be due to the adjustment of sensibly equivalent tensions on the legs of the two sides. As indicated in connection with Fig. 10, the conditions are in fact too complex for calculation; a more significant test is indeed obtainable by measuring geotropic orientation of beetles of other species, and of ants, in which the abdomen is normally not directly in contact with the inclined substratum.

SUMMARY.

The creeping of the beetle *Tetraopes tetraophthalmus* during negatively geotropic orientation shows the angles of orientation (θ) on a surface inclined at α° to the horizontal to be proportional to $\sin \alpha$. The direction of orientation easily suffers temporary reversal to positive as result of handling. Mechanical stability during upward progression should be just possible when $K_1 \cot \alpha = K_2 \sin \theta + K_3 \cos \theta$, the weight of the body being supported on the tripod formed by the legs on either side and by the posterior tip of the abdomen. Lack of this stability produces tensions on the legs through (1) the bilaterally distributed pull of the body mass on the legs, and (2) the torque on the legs due to the weight of the abdomen. The downward gravitational displacement of the tip of the abdomen causes K_2 and K_3 in the preceding formula to be functions of α .

These relations have been tested in detail by shifting the location of the center of gravity, by attaching additional masses anteriorly and posteriorly, and by decreasing the total load through amputation of the abdomen; the latter operation changes the conditions for stability.

Different formulæ are thus obtained (*cf.* earlier papers) for the orientation of animals in which the mechanics of progression and the method of support of the body weight on an inclined surface are not the same. This demonstrates in a direct way that the respective empirical equations cannot be regarded as accidents. The results are in essence the same as that already obtained with young mammals. The diversity of equations required for the physically unlike cases merely strengthens the conception of geotropic orientation as limited

by the tensions applied to the musculature of the body (caterpillars, slugs) or of appendages (beetles, and certain other forms) when the body is supported upon an inclined surface, since equations respectively pertaining to the several instances, and satisfactorily describing the observations, are deduced on this basis.

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SALT ERROR OF INDICATORS DUE TO STANDARD ALKALINE BUFFERS THEMSELVES. II.

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In a previous paper¹ it was reported that there is a big discrepancy between the true alkalinities or pH values of solutions of standard alkaline buffers and of pure sodium hydroxide which give the same actual color with indicator. The discrepancy amounted to a 100 fold error in the determination of the alkalinity in the most extreme case and was very pronounced with numerous other indicators. Evidently there is a salt error caused by the buffer itself.

Communications from Dr. Mansfield Clark raised the following points: the possible extent of the neutralisation of sodium hydroxide by the added indicator, especially if this had not previously been neutralised, and secondly the practicability of the use of pure dilute solutions of sodium hydroxide as a standard.

The purpose of this communication is to present further experimental work in which these suggested errors have been evaluated. It is found that the former investigation is fully confirmed and even with the rigorous technique the large discrepancies are substantially unaltered.

The colorimetric method previously described has been repeated and extended to include determinations with fully neutralised indicators, direct comparison of neutralised and unneutralised indicator being made with the same standard. The concentration of the hydroxyl ion in the solutions of the pure sodium hydroxide used have been checked by the electrometric method and thus their true alkalinity established in the presence of the indicator. The solutions were prepared as before entirely free from carbon dioxide and carbo-

¹ McBain, J. W., Dubois, O. E., and Hay, K. G., *J. Gen. Physiol.*, 1926, ix, 451.

nate, by the use of sodium drippings,² and boiled out conductance water (1.06×10^{-6}). They were used as soon as prepared and discarded. In addition for most of the E.M.F. work, oxygen was removed from the water by bubbling with hydrogen or the water allowed to cool after boiling in an oxygen-free atmosphere. With these precautions higher and far more consistent values have been obtained with the hydrogen electrode than have hitherto been recorded since previous investigators obviously failed with dilute solutions of alkali.

Experimental Method and Materials.

The original method was employed; namely, the direct comparison of colors given by indicators in buffer solutions and those given by pure sodium hydroxide solutions of known concentration and known alkalinity. The buffers used were Sørensen's glycine/sodium hydroxide and Palitzsch's borax/boric acid made according to the directions of Clark.³

The glycol, sodium chloride, borax and boric acid were all of the highest quality obtainable. The sodium hydroxide solutions were standardized by the Bureau of Standards benzoic acid and constant boiling hydrochloric acid (method of Hulett and Bonner⁴).

The indicators used were:

Thymol violet pH = 9.0 – 13.0, B.D.H. (British Drug Houses).

o-Cresol phthalein, pH = 8.1 – 8.9, B.D.H.

Alizarin yellow G. (*p*-nitrobenzeneazosalicylic acid = alizarin yellow) pH = 10 – 12.1, B.D.H.

² The method we have used for the past 18 years at Bristol University in connection with precision work on soaps is to place clean metallic sodium in a nickel gauze suspended in a desiccator over a nickel crucible. The desiccator contains a solution of sodium hydroxide free from carbon dioxide. It is essential merely to use a vacuum desiccator but to have it fitted with well vaselined glass stopper to which is sealed on a U-tube containing a strong aqueous sodium hydroxide filling the bend of the U-tube. In this way it is possible to avoid ingress of carbon dioxide. Solutions prepared in this way exhibited a conductivity 1 per cent greater than the best obtained by other investigators such as collaborators of W. A. Noyes in 1907, see *Z. physik. Chem.*, 1911, lxxvi, 179.

³ Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd ed 1922, 111, 115.

⁴ Hulett, G. A., and Bonner, W. D., *J. Am. Chem. Soc.*, 1909, xxxi, 3907.

Tropæolin O (*p*-benzeneazoresorcinsulfonic acid), pH = 11.1 – 12.7 B.D.H.
 Thymol blue, B.D.H. (Thymolsulfonphthalein).
 Phenolphthalein, "Kahlbaum."
 Phenol red, B.D.H. (Phenolsulfonphthalein).

On calculating the effect of the carbon dioxide in the laboratory it is found that there is enough present in 50 cc. of air to neutralise one half of the same volume of 0.0001N sodium hydroxide and all of 0.00001N. Hence special care was taken in making the solutions,

TABLE I.
Concentrations of Indicators Used.

Indicator	Concentration of solution	Amount used per 100 cc. solution
		<i>drops</i>
Alizarin yellow G.	0.01 per cent in water	10
Universal indicator		10
<i>o</i> -Cresol phthalein	0.02 per cent in alcohol	10
	The same neutralised	10
Thymol blue	0.04 per cent monosodium salt in water	10
	0.04 per cent of indicator in water	10
Phenolphthalein	0.5 per cent in 95 per cent alcohol	6
	The same neutralised	6
	0.1 per cent in 50 per cent alcohol	6
Tropæolin O	0.02 per cent in water	10
Thymol violet		3
Phenol red	0.04 per cent monosodium salt	10
	0.02 per cent monosodium salt	10
	0.02 per cent indicator in water	10

using long necked volumetric flasks as containers and running only one sample from the flask. The samples were quickly transferred to Jena test-tubes of 5/8 inch bore and paraffined corks inserted. The matching is done under a daylight lamp.

The results for the buffer solutions are calculated from the formula $\text{pH} = \log 1/\text{H}^+ = \log \text{OH}^+ \log 1/K_w$. The pH values of the sodium hydroxide are calculated directly from the known normality, assuming these dilute solutions to be wholly dissociated.

The values of K_w used were:

$$K_w \text{ } 18^\circ\text{C.} = 0.72 \times 10^{-14}$$

$$K_w \text{ } 20^\circ\text{C.} = 0.80 \times 10^{-14}$$

If the calculations are made from the mean K_w of the values recorded by Kolthoff⁵ namely, 0.63 at 18°, and 0.71 at 20° the discrepancies here recorded are enhanced. The experimental results of the colorimetric determinations are recorded in Tables I–VII (work by O. E. C.).

TABLE II.

Concentrations and Hence pH of Sodium Hydroxide Solutions Here Found to Give the Same Color As the Standard Sørensen Glycine Buffers at 18°C., Confirming the Observations of McBain, Dubois and Hay (Bristol) Which Are Put First for Each Indicator for Comparison.

Buffer			Sodium hydroxide with					
Composition		Accepted values	Thymol violet		Tropæolin O		Alizarin yellow	
Glycine	NaOH		Bristol	Clark	Bristol	Clark	Bristol	Clark
2	8	$C_{OH} \cdot 0.051$ pH 12.86	0.046 12.82	0.047 12.82				
3	7	$C_{OH} \cdot 0.034$ pH 12.67		0.032 12.65	0.039 12.74	0.035 12.69		
4	6	$C_{OH} \cdot 0.018$ pH 12.40	0.017 12.50	0.021 12.47	0.021 12.47	0.021 12.47		
4.5	5.5	$C_{OH} \cdot 0.0073$ pH 12.10	0.011 12.18	0.011 12.18	0.011 12.18	0.010 12.14	0.0096 12.12	0.010 12.14
4.9	5.1	$C_{OH} \cdot 0.0027$ pH 11.57	0.0058 11.89	0.0042 11.76			0.0065 11.96	0.005 11.84
5	5	C_{OH} pH 11.31						0.004 11.74

Phenolphthalein and *p*-cresol phthalein were dissolved as specified, then titrated with 0.1N sodium hydroxide till distinctly colored. This was used as the neutralised indicator. Thymol blue and phenol red were made up by dissolving the pure dye in water, and also by neutralising with one equivalent of sodium hydroxide according to Clark's directions.⁶ It would seem inadvisable to grind the sodium

⁵ Kolthoff, I. M. and Furman, N. H., *Indicators*, New York, 1926, 248.

⁶ Clark, W. M., *The determination of hydrogen ions*, Baltimore, 1922, 81.

hydroxide and indicator in the air in the mortar because carbon dioxide is taken up so rapidly. Hence, in addition to following Clark, direct neutralisation of indicator was also made.

It will be seen from Table II that the readings in this laboratory closely parallel the readings in the previously published paper in matching thymol violet, alizarin yellow G and tropæolin O, thus in-

TABLE III.

Buffer		Buffer 18*	Thymol blue Bristol 18*	Buffer 20*	Thymol blue (Clark)	
Glycine	NaOH				Neutral	Not neutral
8	2	C_{OH} 0.000016 pH 9.36	0.000099 10.14	0.000016 9.31	0.000032 10.01	0.00012 10.18

Salt error found in the Bristol paper $10.14 - 9.36 = 0.78$ pH.

" " " " this laboratory using indicator 1 equivalent NaOH $10.01 - 9.31 = 0.70$ pH.

Salt error using indicator dissolved in water $10.18 - 9.31 = 0.83$ pH.

TABLE IV.

Buffer		Buffer 18*	o-Cresol phthalein	Buffer 20*	o-Cresol phthalein (Clark)	
Glycine	NaOH				Neutral	Not neutral
7.5	2.5	C_{OH} 0.000036 pH 9.66		0.00024 9.48	0.000098 10.090	0.000099 10.092
8	2	C_{OH} 0.000016 pH 9.36	Bristol 0.000099 10.14	0.000016 9.31	0.000078 9.99	0.000079 9.994

Salt error found in Bristol paper $10.14 - 9.36 = 0.78$ pH.

" " " " this laboratory $9.99 - 9.31 = 0.68$ pH.

dicating that the results are easily reproducible if pure solutions of sodium hydroxide are employed.

It is found that the discrepancy varies according to the manner of preparing the solution of indicator, but that the variation is unimportant compared with the total difference between the pH of the buffer and the pH of the sodium hydroxide solution giving any one color with the indicator. Such a discrepancy of say 0.7 units of pH is an error of fivefold in the measurement of the concentration of hy-

droxyl ion by the usual method of employing these indicators and buffers.

There is no appreciable difference between the results when using *o*-cresol phthalein as such or the same solution titrated with sodium hydroxide until distinctly colored.

In the case of Tables V and VI the figures quoted from the Bristol paper should be revised, especially since the new results were confirmed

TABLE V.

Sørensen buffer. Boric acid.		Buffer 18°	Phenolphthalein 0.5 per cent			Phenolphthal- ein in 1 per cent
Borax	NaCl		Bristol	Neutral	Not neutral. Clark	Neutral
10	0	C_{OH} 0.000012 pH 9.24	0.00058 10.91	0.0003 10.62	0.0003 10.62	0.000092 10.11
9	1	C_{OH} 0.0000092 pH 9.11	0.0005 10.84	0.00027 10.57	0.00027 10.57	
8	2	C_{OH} 0.0000065 pH 8.98	0.00038 10.72	0.00022 10.48	0.00023 10.50	
7	3	C_{OH} 0.0000051 pH 8.84	0.00033 10.66	0.00017 10.37	0.00018 10.40	
6	4	C_{OH} 0.0000036 pH 8.69	0.000285 10.59	0.00013 10.25	0.00014 10.29	

Salt error found in Bristol paper $10.91 - 9.24 = 1.67$ pH (0.5 per cent).

" " " " this laboratory $10.62 - 9.24 = 1.38$ pH (0.5 per cent).

" " " " " " $10.11 - 9.24 = 0.87$ pH (0.1 per cent).

by using borax, half of which had first been washed away, and by using recrystallised borax, and by obtaining a sample of the borax used at Bristol, and by varying the amount of phenolphthalein, and finally by comparing the two original samples of borax electrometrically.

The colorimetric work may be summarised as follows; the serious difference between the alkalinity of the standard buffers and the concentration of pure sodium hydroxide giving the same color upon addition of indicator is confirmed. The differences between buffer

and pure alkali depend upon the indicator and its concentration, being less for less indicator. Neutralising *o*-cresol phthalein and phenolphthalein had little effect but in the case of thymol blue and phenol red neutralisation does make a small difference. The difference in the

TABLE VI.

Buffer. Boric acid		Buffer 18*	Thymol blue. <i>Bristol</i>	Thymol blue. Clark	
Borax	NaCl			Neutral	Not neutral
10	0	$C_{OH} 0.000012$ pH 9.24	0.000296 10.61	0.000076 10.02	0.000098 10.16
9	1	$C_{OH} 0.0000092$ pH 9.11	0.000197 10.44	0.000065 9.95	
8	2	$C_{OH} 0.0000065$ pH 8.98	0.000148 10.31	0.000052 9.86	
7	3	$C_{OH} 0.0000051$ pH 8.84		0.000042 9.77	
6	4	$C_{OH} 0.0000036$ pH 8.69		0.000033 9.66	
5.5	4.5	$C_{OH} 0.0000029$ pH 8.60	0.000099 10.14	0.00003 9.62	
5	5	$C_{OH} 0.0000018$ pH 8.41			
10 to 25 drops were used.					
Salt error found in Bristol paper			10.61 - 9.24 = 1.37 pH.		
" " " " this laboratory			10.02 - 9.24 = 0.78 pH*		
" " " " " "			10.16 - 9.24 = 0.92 pH.†		

* Indicator plus 1 equivalent NaOH.

† Pure indicator.

alkalinity of the buffer and the concentration of sodium hydroxide of the same color, is shown in graphical form in the broken lines in Fig. 1. As will be shown in the following section dealing with the electrometric measurements most of the discrepancy must be attributed to salt

error caused by the alkaline buffers themselves. This is represented by the solid lines in Fig. 1 (see later). Between pH's 12 and 13 with glycine buffers, alizarin yellow G, thymol violet and tropæolin O are free from it but at pH 9.4 it amounts to about 0.6 pH and 0.8 pH units

TABLE VII.

Buffer. Boric acid		Buffer 18°	Phenol red. Bristol	Phenol red (Clark)		
Borax	NaCl			0.04 per cent. Neutral	0.02 per cent	
					Neutral	Not neutral
3	7	C _{OH} 0.00000087 pH 8.08	0.000124 10.24	0.000097 10.13	0.000062 9.92	0.00015 10.33
2.5	7.5	C _{OH} 0.0000006 pH 7.94		0.000090 10.12	0.000058 9.91	
2.3	7.7	C _{OH} 0.00000055 pH 7.88	0.000099 10.14	0.000093 10.11	0.000055 9.88	
2	8	C _{OH} 0.00000042 pH 7.78		0.000085 10.07	0.000051 9.85	
1.5	8.5	C _{OH} 0.00000029 pH 7.60	0.000046 9.80		0.000046 9.81	
1.0	0.9	C _{OH} 0.00000016 pH 7.36	0.000025 9.54	0.000055 9.88	0.000038 9.72	

Salt error found in Bristol paper 9.80 - 7.60 = 2.20 pH.

" " " " this laboratory 10.07 - 7.78 = 2.29 pH.*

" " " " " " 9.85 - 7.78 = 2.07 pH.†

" " " " " " 10.33 - 7.78 = 2.55 pH.‡

* Phenol red (0.04 per cent) plus NaOH.

† " " (0.02 per cent) plus NaOH.

‡ " " (pure indicator).

for *o*-cresol phthalein and thymol blue respectively. The worst case occurs with borate buffers at pH 7.3 with phenol red where the discrepancy is $2\frac{1}{2}$ pH units. In general the error is negligible in strong alkali such as pH 13 and increases steadily and rapidly as the alkalinity diminishes.

Part II. Electrometric Determinations.

Proof that the colorimetric work is not illusory is obtained by electrometric determinations of the actual alkalinity of the pure sodium hydroxide itself, before and after adding the indicators.

Leeds and Northrup's best standard equipment was used, employing a Clark's cell, a 0.1N calomel electrode and a saturated potassium

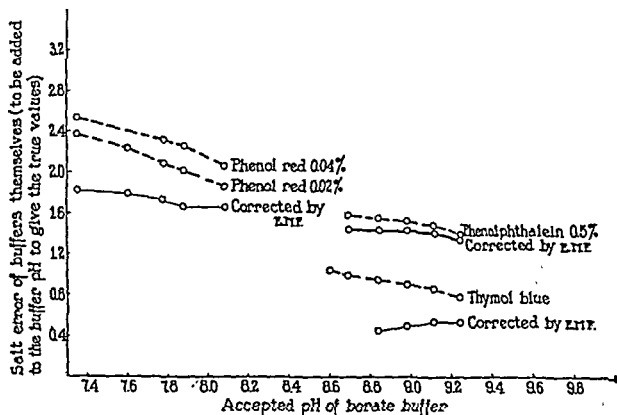
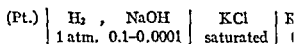


FIG. 1. Comparison between the pH values of standard borate buffers and the concentration and true alkalinity of aqueous sodium hydroxide which give the same color with an indicator. The broken lines show how much greater (in pH units) is the concentration of sodium hydroxide used above that of the hydroxyl ion in the buffer; the solid lines show the difference in true pH (corrected for any action of the indicator upon the dilute sodium hydroxide); that is, the salt error caused by the alkaline buffer itself.

chloride salt bridge and special care was taken to avoid contamination of the sodium hydroxide by the potassium chloride. A number of calomel electrodes were used in each measurement as a check and measurements were not recorded where there was a difference of a few tenths of a millivolt.

The cell measured was:



The pH was calculated from the following equations:

$$\frac{\text{E.M.F. (observed)} - E_h \text{ (calculated electrode)}}{0.00019837 \text{ T}} = \log 1/H^+ = \text{pH, Clark, p. 153}$$

Corrected for pressure

$$\frac{\text{E.M.F.} + E \text{ (bar)} - E_h \text{ (calculated)}}{0.00019837 \text{ T}} = \log 1/H^+ = \text{pH, Clark, p. 161.}$$

By proper adjustment of the Clark cell it is possible to keep the pressure at the hydrogen electrode at atmospheric pressure. The corrections to be applied to each of the actual working pressures are obtained from the graph of the corrections listed by Clark (p. 409). They range from 0.2 to 0.4 millivolts.

Table VIII presents the data for pure sodium hydroxide solutions. Series A are values obtained by O. E. Clark in 1927; series B are values obtained by M. E. Laing in 1928. The voltages in the final series, B, are higher because additional precautions were taken to exclude oxygen from the solutions, since oxygen always lowers the potential found with the hydrogen electrode and because a specially high sensitive galvanometer, Type HS was used for the most dilute solutions.

The only measurements in the literature of this series of dilutions seem to be those of Lorenz and Mohn⁷ and the value for 0.1N given by Sørensen. These earlier values are lower in the dilute concentrations and show an excessive falling off, obviously due to the neutralisation of their sodium hydroxide by the carbon dioxide of the air.

The values were -1.096, -1.045, -0.951 at 30° and 0.1* -1.0891 at 18°.

TABLE VIII.

E. M. F. Data for Solutions of Sodium Hydroxide.

Series A			Series B	
Concentration	E.M.F.	pH	E.M.F.	pH
0.1	1.094	12.85	1.0965	13.0
0.01	1.041	12.06	1.0435	12.14
0.001	0.9807	10.94	0.9876	11.18
0.0001	0.9156	9.91	0.9230	10.07

⁷ Lorenz, R., and Mohn, A., *Z. physik. Chem.*, 1907, lx, 422.

* Sørensen, S. P. L., *Electrochem. Z.*, 1909, xxi, 131.

The E.M.F. of a concentration cell involving two solutions of sodium hydroxide with no diffusion is $\frac{RT}{F} \ln c_1/c_2$ and the change in E.M.F. at the working temperature 23° should be 58.7 millivolts and at 20° 58.1 for a tenfold drop in hydroxyl ion if dissociation is complete. In series (a) the change in millivolts found for successive tenfold dilutions are 53, 60 and 64 and the final series (b) 53, 56 and 65. This means substantial accuracy in spite of the fact that the difficulty of the measurements increases with dilution. For example Lorenz and Mohn's value for 0.001 had dropped by no less than 94 millivolts from that for 0.01 and ours are the only successful measurements for N/10,000.

The results of the corresponding electrometric determinations in the presence of indicators are recorded in Tables IX-XII. In both series (a and b), 10 drops of indicator are added to 10 cc. of freshly diluted sodium hydroxide.

TABLE IX.

pH Determinations of Pure Aqueous Sodium Hydroxide Alone Compared with Similar Solutions Containing Neutralised and Unneutralised Thymol Blue.

Concentration of solution	Temperature	E.M.F.	pH	Reduction in pH due to indicator
<i>Series (a)</i>				
0.001 NaOH	23	0.9800	10.94	
0.001 NaOH + neutralised T.B.	23	0.9725	10.82	0.13
0.001 NaOH + unneutralised T.B.	23	0.9716	10.80	0.14
0.0001 NaOH	22	0.9131	9.34	0.30
0.0001 NaOH + neutralised T.B.	22	0.8970	9.53	
0.0001 NaOH + unneutralised T.B.	22	0.8881	9.41	0.42
<i>Final series (b)</i>				
0.01 NaOH	20	1.0435	12.14	0.04
0.01 NaOH + neutralised T.B.	20	1.0409	12.10	
0.001 NaOH	20	0.9876	11.18	0.04
0.001 NaOH + neutralised T.B.	20	0.9850	11.14	
0.0001 NaOH	20	0.9230	10.07	0.19
0.0001 NaOH				

The neutralised indicators of series (a) were kept in stoppered flasks. They were likewise used in work preparatory to the final series (b). The pH difference between pure 0.01 and 0.001N sodium hydroxide on the one hand and these solutions containing thymol blue on the other were -0.09 and -0.15 respectively, giving practically the same result as in series (a).

It should be noted that the pH differences of final series (b) are half these of series (a) due to improved technique in handling these dilute solutions.

Table X records final series (b) with neutralised and unneutralised 0.02 per cent phenol red indicator.

TABLE X.

Effect of Neutralised and Unneutralised Phenol Red upon Solutions of Sodium Hydroxide.

Concentration of solution	Temperature	E.M.F.	pH	Lowering of pH due to indicator
<i>Final series (b)*</i>				
0.0026 NaOH	20	1.0090	11.55	
0.0026 NaOH + neutralised P.R.		1.0077	11.52	0.02
0.001 NaOH	18	0.9854	11.22	
0.001 NaOH + neutralised P.R.		0.9845	11.20	0.02
0.0003 NaOH	20	0.9566	10.65	
0.0003 NaOH + neutralized P.R.		0.9438	10.43	0.22

* Fresh 0.0001 NaOH neutralised P. R. as used in series (a) gave pH difference of 0.20.

Table XI contains the data for solutions of sodium hydroxide with and without neutralised and unneutralised phenolphthalein.

The indicator is made with 0.10 gm., of Kahlbaum's solid indicator in 50 cc. each of water and alcohol, both being boiled out immediately before use. Two procedures were used in neutralising the phenolphthalein (not following Clark). Either the phenolphthalein is titrated direct with 0.1N sodium hydroxide until distinctly pink or the

calculated amount of solution of sodium hydroxide required to make the monosodium salt is added.

TABLE XI.

Effect of Neutralised and Unneutralised Phenolphthalein upon Solutions of Sodium Hydroxide.

Concentration of solution	Temperature.	Z.M.V.	pH	Lowering of pH due to indicator
<i>Series (a)</i>				
	°C.			
0.001 NaOH	24	0.9807	11.01	
0.001 NaOH + neutralised Ph.		0.9787	10.99	0.02
0.001 NaOH + unneutralised Ph.		0.9770	10.96	0.05
0.0001 NaOH	22	0.9140	10.91	
0.0001 NaOH + neutralised Ph.		0.8874	9.46	0.45
0.0001 NaOH + unneutralised Ph.		0.8868	9.45	0.46
<i>Final series (b)*</i>				
0.01 NaOH	19	1.0406	12.13	0.01
0.01 NaOH + neutralised Ph.		1.0396	12.12	
0.0026 NaOH	20	1.0090	11.55	0.02
0.0026 NaOH + neutralised Ph.		1.0076	11.53	
0.001 NaOH	18	0.9857	11.22	0.02
0.001 NaOH + mono Na Ph.		0.9845	11.20	
0.0003 NaOH	20	0.9565	10.65	0.24
0.0003 NaOH + neutralised Ph.		0.9427	10.41	
0.0001 NaOH	19	0.9227	10.10	0.20
0.0001 NaOH + neutralised Ph.		0.9114	9.90	

* Other results preparatory to series (b) for 0.001 NaOH plus sodium salt pH difference = 0.03; for 0.0026 NaOH plus 2 drops 0.5 per cent indicator pH difference = 0.002.

DISCUSSION.

Granted that we have sufficiently shown that we have succeeded in preparing dilute solutions of sodium hydroxide of substantial purity

TABLE XII.

Data Using Alizarin Yellow G and 0.05 Molar Borax.

Concentration of solution	Temperature	E.M.F.	pH	Difference in pH
	°C.			
0.001 NaOH	18	0.9854	11.22	
0.001 NaOH + alizarin yellow G		0.9825	11.17	-0.05
0.0001 NaOH	18	0.9227	10.10	
0.0001 NaOH + alizarin yellow G		0.9093	9.90	-0.20
0.05 borax	19.8	0.8757	9.25	
0.05 borax		0.8764	9.27	+0.02

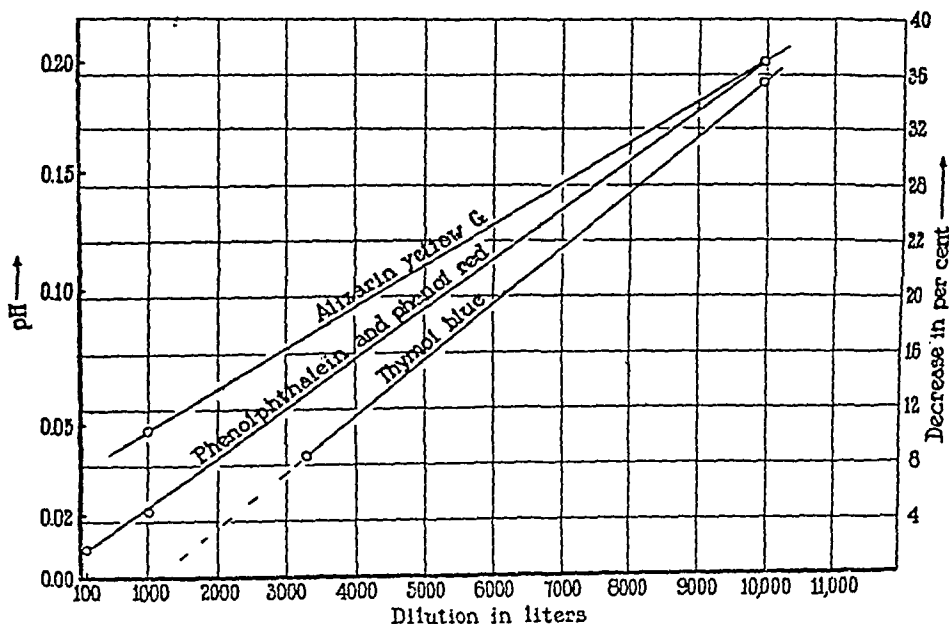


FIG. 2. Correction diagram showing extent to which the true pH of pure dilute aqueous sodium hydroxide is lowered by the addition of indicators.

the main conclusion of our colorimetric work stands; namely, that there is a large salt error inherent in the standard buffers themselves.

It is true that there is a slight neutralising effect of the indicators but these are such weak acids and their salts are so extensively hydrolysed as to make this a distinctly minor effect not exceeding 0.2 pH in the most dilute solutions used. It is therefore usually much less than the error of colorimetric determinations (0.2 pH corresponds to an error of 60 or -37 per cent).

The extent of the relatively slight neutralising action of the four indicators, alizarin yellow G, phenolphthalein, phenol red and thymol blue is shown in Fig. 2 for various dilutions (in liters) of pure sodium hydroxide. The neutralisation diminishes the alkalinity of the sodium hydroxide approximately proportional to the dilution. Two scales are provided in Fig. 2 so that the effect on the indicator in diminishing the true alkalinity of the sodium hydroxide as determined by the hydrogen electrode may be read either in pH units or in per cent decrease in concentration.

It will be seen that all four indicators produce almost the same effect in $N/10,000$ sodium hydroxide, namely 0.2 pH units decrease of alkalinity but that for phenolphthalein and phenol red this has fallen to the negligible value of 0.02 pH units for $N/1000$ sodium hydroxide being still less for thymol blue and even for alizarin yellow G being only 0.05 pH units.

These corrections are negligible in comparison with the serious salt errors found, as can be seen from Fig. 1, where the broken lines recorded the actual concentration of hydroxide giving the same color as the buffer and the corresponding solid lines are corrected by means of Fig. 2.* For example phenol red still shows as much as two whole pH units salt error, due to the borate buffer, that is, an error of 99 fold.

SUMMARY.

Previous results of the comparison of colors given by indicators in alkaline buffers and pure aqueous sodium hydroxide have been repeated and confirmed. The electrometric determinations show that the sodium hydroxide was pure and gave theoretical values for the

* It may be mentioned that the results in Fig. 2 have been made use of in the calculation of the alkalinity of a comprehensive set of data for soap solutions by McBain and Hay (now communicated to the *J. Chem. Soc.*). Linear extrapolation has been used for dilutions greater than 0.0001 N .

concentration of hydroxyl ion. The slight but distinct neutralising effect of dilute solutions of alkali has been measured electrometrically and the allowances to be made are recorded graphically. It is found that whereas alizarin yellow G, tropæolin O and thymol violet may be used without appreciable error (in accordance with our previous communication) the grave discrepancies remain for phenolphthalein, *o*-cresol phthalein and thymol blue and phenol red which must be ascribed to salt error in the alkaline buffer itself.

ANNOUNCEMENT

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THE CATAPHORETIC VELOCITY OF MAMMALIAN RED BLOOD CELLS.

By HAROLD A. ABRAMSON.

(From the Laboratory of Research Medicine, Medical Clinic, the Johns Hopkins University, Baltimore.)

(Accepted for publication, March 20, 1929.)

INTRODUCTION.

The cataphoretic velocity of the red blood cells of different mammals is determined by certain surface characteristics of the cells and hence is of value in studying the constitution of the cell surface. The correlation of these values with the zoological classification of the animals may have phylogenetic or ontogenetic significance. The cataphoretic velocities of the red blood cells of a group of mammals is the subject of this communication.

HISTORICAL.

There have been a great many experiments dealing with the sign of charge of red cells and the order of speed with which the red cells of different mammals move in an electric field. These experiments, however, must be considered for the most part as qualitative, and when fine distinctions are to be drawn, very frequently erroneous. Kozawa (1) noted that the red cells of different animals could be brought to an isoelectric point at a pH characteristic for each animal, the pH decreasing in the order

Rabbit > Guinea pig > Cat, Human > Dog > Pig.

Less acid was required if the suspensions were allowed to stand. It was evident to Kozawa that some change with time took place in the suspensions which altered the surface of the red cells. Kosaka and Seki (2) have also attempted to compare the red cells of different animals. In 0.9 per cent NaCl (the pH is not given) they found the qualitative series, (order of increasing velocity)

Rabbit < Pig < Guinea pig < Man < Mouse < Rat, Cat, Dog.

If the suspending fluid was 9.5 per cent saccharose instead of saline the order just cited was reversed to

Cat, Dog < Man < Rat < Guinea pig < Pig < Rabbit.

Here again there is no mention of degree of acidity. Kosaka and Seki state further that the red cells of the rabbit in serum move to the cathode. Such data show that there probably are differences, but they cannot be used quantitatively. Kozawa's observations, namely, that the surfaces of red cells in acid media undergo an important change easily perceptible by the method of cataphoresis were confirmed and extended by Eggerth (3) and also by Netter (4). Eggerth also found that human cells at pH 5.2 and below, particularly in buffered sugar solutions, show a marked change in velocity on standing. Netter found the same for ox and horse red cells. This change occurred whenever cells remained for some time in a solution of low salt concentration. It is evident that experiments which deal with differences in red cells of different animals in acid solutions are not easily interpreted because the acid produces an unstable system at the red cell surface. One might, perhaps, speak of a "dying red cell" with death or "equilibrium" at the end of 2 hours immersion—the time at which Netter considered "equilibrium" to be present. One may properly compare red cells of different animals in a given medium only if there is no change with time. Netter found that horse red cells in serum and in buffered saline solutions moved slightly faster than ox cells in the same medium. Bernardi (5) has found the following series:

Rabbit < Cat < Guinea pig < Man < Pig.

Much of the data cited above have been obtained under uncertain experimental conditions and with practically complete disagreement amongst the various authors.

Freundlich and Abramson (6) have found that red and white cell velocity in serum did not vary very much in 10 different adult horses, although differences between red cell and white cell velocity were marked. Abramson (7) has published similar data for red and white cells in plasma of 7 different horses.

METHODS.

The method of measurement and of calculation of absolute cataphoretic velocity was performed in the manner described by Abramson (8). The apparatus employed was a modification of that first published by Northrop and Kunitz (9) and Kunitz (10). A single apparatus was used throughout. The values of mobility for this cell corresponded within 9 per cent of values determined by means of a cemented cell of the same general construction, but of different dimensions. Mobility values found by Abramson (11) for quartz particles covered by albumin in this cemented cell have been checked by the streaming potential measurements of Briggs (12) on similar surfaces of quartz membranes covered with the same protein. The velocities here given for red cells, having been checked directly and indirectly, should represent absolute mobilities certainly within 10 per cent. It will later appear that smaller differences may, on occasion, be extremely simply estimated.

The blood cell suspensions were made up as follows. One drop of freshly drawn blood (in the capillary portion of a white cell pipette) was added to 25 to 50 cc. of $M/15$ phosphate buffer of $pH = 7.35 \pm 0.03$ so that an exceedingly dilute suspension of blood cells was obtained. As will appear later, the volume of the blood added, within reasonable limits, does not interfere with the constancy of cataphoretic velocity of the blood cells. The buffer employed had a specific resistance of 126 ± 1 Ohms at $25^{\circ}C$. Measurements of cataphoresis were made at room temperature, which usually varied between 22° and $27^{\circ}C$. The values in the tables are corrected to 25° by assuming that the temperature coefficient of the cataphoresis of the red cell in the medium employed is 0.02 per degree Centigrade.

The type of cataphoresis cell used is readily cleaned with cleaning mixture between measurements. This precaution was not taken, however, as experiment proved it to be unnecessary. Running tap water was drawn between measurements for 2 minutes through the cell by means of a suction pump. If it be desired to study the effect of short periods of time on mobility the cell can be turned upside down, and the particles resuspended.

EXPERIMENTAL.

The data discussed in the historical section were obtained for the most part under conditions which did not lead to a steady state without necessitating a destruction of the red cell structure. Before the cells of different animals could be compared, therefore, it was essential that a medium be employed which had no destructive effect on the cell. M/15 phosphate buffer of pH 7.4 was chosen. Table I gives the effect of time of standing on mobility. It is evident that for a large variety

TABLE I.

The Effect of Standing Time on Velocity.

There is no significant change under the experimental conditions.

Animals	Velocity at first <i>μ per sec. per volt per cm.</i>	Velocity after standing <i>μ per sec. per volt per cm.</i>
Man 1.....	1.21	1.12 (12 hrs.)
“ 2.....	1.22	1.22 (48 hrs.)
“ 3.....	1.23	1.20 (18 hrs.)
“ 4.....	1.30	1.29 (18 hrs.)
“ 5.....	1.20	1.15 (24 hrs.)*
“ 6.....	1.28	1.18 (24 hrs.)*
Opossum.....	1.05	1.11 (24 hrs.)
Dog.....	See Table III	1.55 (12 hrs.)
Cat.....	See Table III	1.39 (12 hrs.)
Sloth.....	0.97	1.00 (24 hrs.)
White rat.....	See Table III	1.43 (24 hrs.)

* Sickle cell anemia.

of animals (man, dog, cat, sloth, opossum, rat) the cataphoretic velocity remains unchanged in this medium for at least 24 hours. A second matter to be considered was the effect of further dilution and of repeated washing of the red cell suspensions. The suspensions studied, as Table II demonstrates, were as dilute as necessary to obtain constant values. Washing with large volumes of buffer produced no significant change in the velocities of rabbit and human cells. It is to be noted that almost the same constant value was obtained if washed cells were hemolyzed by diluted hypotonic buffer and cells subsequently added to the hemolytic mixture (which had been again made isotonic). This confirms a similar observation made by Eg-

gerth: that red cells adsorb little if any hemoglobin in media at this reaction.

The Cataphoretic Velocity of Normal Adult Human Cells.

A series of 10 normal white adults gave a mean velocity of 1.31 ± 0.02 μ per second per volt per centimeter. Both sexes were about equally represented and the ages varied from 19 to 53 years.¹ The mean of 10 normal adult Negroes velocity was $1.30 \pm 0.05 \mu$ per second per volt

TABLE II.

10 cc. of a dilute suspension of red cells whose volume per cent was less than 2 per cent were washed at least 5 times with 10 cc. of N/15, pH 7.4 phosphate buffer. The residual volume was about 0.3 cc. The data are not corrected to 25°C. There is no easily perceptible change.

Exp.	Animals	Velocity before washing	Velocity after washing	Remarks
		μ per sec. per volt per cm	μ per sec. per volt per cm.	
1	Man	1.14	1.20	Hypertension Nephritis Secondary anemia
2	Man	1.27	1.29	
3	Man	1.31	1.31	
4	Rabbit	0.50	0.52	
5}	Human cells washed as above, then hemolyzed; similarly washed cells then added. See text	See table for limits of normal	1.20	
6}			1.34	

per centimeter. Three members of the Yellow race possessed similar velocities in comparable experiments. This is indirectly confirmatory of the observations of Schroeder* who noted that there were no differences in speed amongst red cells of the various Landsteiner blood groups (Table III). While it is relatively simple to get values corresponding within 3 to 5 per cent on the same day, errors of meas-

* Schroeder, *Pflug. Arch. f. d. ges. Physiol.*, 1926, ccxv, 32.

¹ Another series of 10 white adults in a buffer having a slightly greater salt content gave a value of 1.22 ± 0.02 per second per volt per centimeter.

urement from day to day lead to wider variations as is evident from Table IV where the measurements were made during several weeks

TABLE III.

The Cataphoretic Velocity of Mammalian Red Cells at pH 7.35 in M/15 Phosphate Buffer.

The velocity of a human control is given only when small differences are to be considered.

Order	Animal	Number of animals investigated	Mn. observed velocities <i>μ per sec. per volt per cm.</i>	Average deviation	Velocity of human control <i>μ per sec. per volt per cm.</i>	Remarks
Primate	Man (white)	10	1.31	± 0.02	1.23	2 young monkeys
	Man (negro)	10	1.30	± 0.05		
	Monkey (<i>Macacus rhesus</i>)	4	1.25	± 0.02		
Carnivor	Dog	3	1.68	± 0.03	1.32	Fairly rapid hemolysis
	Cat	3	1.40	± 0.01		Fairly rapid hemolysis
Ungulate	Pig	5	0.98	± 0.03		Rapid spontaneous hemolysis
Rodent	Rabbit	5	0.55	± 0.05		Included 2 young rabbits
	Guinea pig	6	1.11	± 0.02	1.26	4 white mice
	Mouse	5	1.35	± 0.06		4 white rats
	Rat	5	1.45	± 0.02	1.31	
Edentate	Sloth	1	0.97			Two-toed sloth One series of measurements
Marsupial	Opossum	1	1.07	$\pm 0.02^*$		Adult, male

* Two series of measurements.

on a single individual. Although the mean is practically the same within the limits of error, it is evident that for fine differences in velocity between different animals a comparison must be made with a

control under as similar experimental conditions as possible. The ideal arrangement would be to study cells from different animals simultaneously in the same suspension. As will appear shortly, this is possible.

Normal White Infants.—Table V demonstrates that the cataphoretic velocity of the red cell in the new-born white infant is similar to that in the adult. During the extra-uterine life of the white race, therefore,

TABLE IV.

Measurements Repeated over 7 Weeks Showing Variation in Control.

Date	Velocity
	μ per sec. per volt per cm.
Oct. 18	1.29
23	1.26
24	1.22
26	1.30
28	1.21
30	1.19
Nov. 2	1.25
6	1.31
7	1.37
11	1.28
12	1.37
16	1.29
17	1.29
21	1.26
26	1.35

Mn = 1.28 ± 0.04 .

the surface of the red cell remains, on the whole, the same. It is evident that the problem may be extended to the study of changes during intra-uterine development.

Mammals.—The data given in Table III clearly show that very wide differences in the cataphoretic velocity of the red cells of different mammals exist. The absence of an effect of age in humans has been noted. Cells of young monkeys and young rabbits had the same speed as the adults. The mean value of 0.55 per second per volt per centimeter found for the rabbit is but one-third of the speed of dog cells. In the series here presented the following order is found.

Rabbit < Sloth, Pig < Opossum, Guinea pig < Man, Monkey < Mouse, Cat,
 (0.55) (0.97) (0.98) (1.07) (1.11) (1.31) (1.33) (1.40) (1.39)
 Rat < Dog
 (1.45) (1.65)

The number under each type of animal is the value in μ per second per volt per centimeter taken from the tables but corrected for the value of the controls when small differences are present. Thus for monkey the speed found was 1.25 per second. The control (human) was 1.23 per second. Taking 1.31 per second as the standard speed, the speed for the monkey becomes 1.33μ per second.

TABLE V.
Normal White Infants.

Baby	Age	Velocity <i>μ per sec. per volt per cm.</i>
Series I:		
No. 1.....	9 days	1.28
Control adult.....		1.22
Series II:		
No. 2.....	5 days	1.23
No. 3.....	3 hrs.	1.18
No. 4.....	2 days	1.23
No. 5.....	1 hr.	1.18
No. 6.....	1 day	1.27
No. 7.....	1 day	1.22
Control adult.....		1.21
Mn. infants (Series II).....		1.25

It is to be emphasized that these values are for the particular experimental conditions described above. It is known that changes in pH or of salt concentration or valence can produce changes in the cells of a single type of animal greater than the variations described here for the cells of different animals.

This series has been confirmed by comparing red cells of different animals in the same medium at the same time. If rabbit cells are mixed with human cells, it is quite striking to see the human cells, which move more than twice as fast as those of the rabbit overtake

and pass the rabbit cells. In this manner it can be clearly demonstrated that mouse cells, for example, move faster than human cells. The smaller mouse cells may be easily distinguished from human cells and the greater speed of the former easily perceived. The presence of high concentration of electrolytes makes measurements less precise than in more dilute solutions because of the low particle speed and because of the increased electroendosmotic flow, with turbulence more readily occurring. The differences between mouse and human cells which have been measured in successive measurements are at the limits of the experimental error. It was consequently observed with satisfaction that these slight differences which had been measured could be confirmed by direct simultaneous observation. The fact that small amounts of heterologous plasma does not change the red cell surface to an appreciable extent under these conditions is in keeping with the previous observation that red cells can migrate through gelatin serum sols and gels without adsorbing appreciable amounts of protein (13). These experiments can be confirmed by other striking experiments which demonstrate that the red cell preserves its surface integrity. Human red cells were added to a suspension of rabbit cells in rabbit blood (diluted 1:4 with isotonic glucose). In this mixture, which contained about 2 per cent of rabbit plasma proteins, human cells moved about twice as fast as the rabbit cells. And similarly, in the converse experiment, rabbit cells in human serum (diluted 1:6 with isotonic glucose) migrated with about one-half the speed of human cells. This fact may be of use in determining the fate of intravenously injected heterologous red cells. A simple experiment (Table VI) demonstrates that while cholesterol and quartz adsorb gelatin from dilute solution in $M/15$ phosphate buffer at $pH = 7.35$, the red cell even after 24 hours has not changed its cataphoretic velocity. The red cell surface is, therefore, here also practically unaffected by the proteins present.

These experiments do not demonstrate that union of red cell with heterologous serum protein does *not* take place at all. It seems likely, in view of preliminary experiments dealing with this point, that some combination does take place in high concentrations of serum with certain components of normal inactivated serum. Whether or not the reaction is similar to that which is supposed to accompany sensi-

tization is under investigation and will be described in a future communication.

Kosaka and Seki have noted that suspending the cells in isotonic sugar solutions reverses the order of magnitude of mobility. Table VII gives data in disagreement with these findings. The velocity

TABLE VI.

The Effect of Gelatin on the Cataphoretic Velocity of Quartz, Cholesterol and Red Blood Cells in pH 7.35 M/15 Phosphate Buffer.

Particle	Cataphoretic velocity	
	Without gelatin	With gelatin
	μ per sec. per volt per cm.	μ per sec. per volt per cm.
Human red cell.....	-1.31	-1.27 (after 24 hrs. 1.23)
Quartz.....	-3.55	-0.39 approximate
Cholesterol.....	-2.25	-0.35 approximate

TABLE VII.

Red Cells Suspended in Isotonic Dextrose Solution and Dilute Phosphate Buffer (pH = 6.8).

Each value is the mean of two determinations on two animals.

Animal	Velocity	Remarks
	μ per sec. per volt per cm.	
Rabbit.....	1.27	Marked hemolysis
Pig.....	2.09	
Man.....	2.33	
Mouse.....	2.60	

order Rabbit < Pig < Man < Mouse is, as in pure phosphate buffer, also to be found in suspensions of the red cells of these animals in phosphate buffered isotonic sugar solutions.

There is no relationship between our series and the ease with which hemolysis occurs spontaneously. Pig cells are most fragile in M/15 phosphate buffer. Human and rabbit cells hardly hemolyzed at all. Cat and dog cells show a marked tendency to hemolyze.

There was no agglutination within 24 hours; nor did slow centrifugation produce agglutination.

TABLE VIII.

Patient	Age	Race	Sex	Clinical diagnosis	Velocity
					μ per sec. per volt per cm.
1 M.	19	W	F	9 mos. pregnancy	1.35
2 H.	19	W	F	9 mos. pregnancy	1.31
3 P.	34	W	F	9 mos. pregnancy	1.31
4 C.	19	W	F	9 mos. pregnancy	1.30
5 Mc.	26	W	F	Secondary anemia	1.34
6 J.	42	W	M	"Cured" (liver diet) Pernicious anemia	1.30
7 A.	32	B	F	Severe secondary anemia	1.12 (control 1.25)
8 M.	52	W	M	Nephritis; hypertension mild secondary anemia	1.34
9 P.	52	B	F	Carcinoma of colon; mild secondary anemia	1.17 (control 1.26)
10 S.	7	B	M	Sickle cell anemia	1.28
11 J.	8	B	F	Sickle cell anemia	1.20
12	3	B	M	Sickle cell anemia	1.28
13 H.	58	W	M	Pernicious anemia	1.20 (cells of different shape have a same speed)
14 F.	30	W	M	Severe secondary anemia (6 transfusions during 10 wks. previous to admission)	1.25
15 M.	20	W	F	Severe HgCl_2 poisoning. Very severe anemia (citrate transfusion previous day)	1.24
16 M.	45	W	F	Pernicious anemia carcinoma. Very severe anemia	1.35 (transfusion day before. Normal cells in blood suspension)

The approximate ζ -potential may be obtained by multiplying the values given in the table by the factor 13. The rabbit cells, the lowest of the series, have a ζ -potential of about 7 millivolts, while the dog cells

ζ -potential is about 21 millivolts. (Assuming dielectric constant = 80, viscosity = 0.01 for M/15 phosphate buffer at pH = 7.35).

The Red Cell in Pregnancy.

In four cases of pregnancy at term, the cataphoretic velocity of the red cells was normal. This suggests that red cells of pregnant women suspended in citrate (as in the sedimentation test) would have the same mobility as normal women, and that there is here no direct relationship between cataphoretic velocity and sedimentation velocity. This viewpoint is well borne out by related data of Netter. Netter found that horse red cells migrate very slightly faster than ox cells. Horse cells clump quickly and settle out almost at once. Ox cells on the other hand are noted for their stability in suspension without clumping. There is here evidently no relationship between low values of mobility and aggregation. The experiments of Northrop and Freund (14) and of Oliver and Barnard (15) should be consulted in this connection.

The Diseases of the Blood.—A short series of primary and secondary anemias were studied. In primary and secondary anemias, with the exception perhaps of Patient 7 A., no significant change in cataphoretic mobility has been observed. Three cases of sickle cell anemia had practically normal velocities. It is very striking to see all the cells, in anemias where extreme variations in size, shape and hemoglobin content of the cells exist, migrate with practically the same speed. Patient 16 M. had a transfusion the day previous to the examination. There were many normal, probably transfused, red cells to be seen moving with the same velocity as the distinctly abnormal cells. These observations also again demonstrate the obstinacy with which the red cell maintains the general integrity of its surface in spite of most varied circumstances.

DISCUSSION.

The recent experiments of Abramson (13), (16) in collaboration with Freundlich and with Michaelis have made it clear that neither the size nor the shape of microscopic particles suspended in electrolyte-containing media influences the cataphoretic velocity. The differences in red cell velocity given in the preceding statement are, there-

fore, almost certainly representative of the surface constituents of the different red cells. With this in mind the data presented may be discussed.

It is well known that the chemical make-up of the surface of the microscopic particle influences its cataphoretic velocity. And it is of importance to be able to ascribe the variations in velocity found amongst mammalian red cells to a correlated series of changes in the structure of the surface layers. It is more or less customary to consider the red cell surface as hydrophobic. The data of the Mudds is most pertinent and interesting (17). Yet Netter has proposed that the surface of red cells of the horse and the ox, for example, may be composed chiefly of protein. He concluded from cataphoretic experiments that the former was made up primarily of globulin while the latter was chiefly albumin. It is difficult to evaluate Netter's conclusions for his data were primarily obtained in acid solutions where, we have seen, secondary changes always occur. The large differences in mobility found amongst the various mammals, the speed of the rabbit cells of the order 0.55μ per second per volt per centimeter at the one extreme and the dog cells 1.65μ per second per volt per centimeter, at the other extreme, indicate that it is probably not strictly permissible to speak of a characteristic red cell surface in general. The speed of the rabbit cells is very close indeed to the order of magnitude of the speeds found for inert particles covered with protein. The dog cells, on the other hand, are very near the speed of 2.2μ per second per volt per centimeter given here for cholesterol. It is not intended to suggest that cholesterol is a constituent of the red cell surface but rather that substances of a somewhat similar constitution plus protein could account for the high mobility of the dog cell, with mixtures of protein plus this substance or substances producing the intermediate velocities. It may also be mentioned that there is scarcely any quantitative data on the absolute mobility of the plasma proteins and the common lipids.

Grouping the animals used according to order shows clearly that zoological Order and cataphoretic velocity do not invariably go hand in hand. Thus for the Rodents, the rabbit, guinea pig and mouse differ widely, the mouse cells having velocity values much closer to those of the Primates and Carnivora. The most striking observation

is the slow speed in the case of the rabbit cells compared with any other of the animals investigated.

The human red cell when examined in a suitable medium will probably be an excellent particle for the checking of the calibration of cataphoresis cells.

SUMMARY AND CONCLUSIONS.

1. No significant change with time (to 24 hours) in the cataphoretic velocity of certain mammalian red cells occurs when the cells are suspended in M/15 phosphate buffer at pH = 7.35. Neither successive washings nor standing effect a change.

2. In M/15 phosphate buffer at pH 7.35 ± 0.03 the following order of red cell velocity has been obtained. The numbers in parenthesis are μ per second per volt per centimeter.

Rabbit	<	Sloth,	Pig	<	Opossum,	Guinea pig	<	Man,	Monkey	<	Mouse,	Cat,
(0.55)		(0.97)	(0.98)		(1.07)	(1.11)		(1.31)	(1.33)		(1.40)	(1.39)
						Rat	<	Dog				
						(1.45)		(1.65)				

The order, though not the absolute values, was the same in buffered isotonic dextrose. Human and rabbit cells showed similar differences when both were studied simultaneously in the serum of either. Under these conditions, there is no apparent relationship between zoological Order and cataphoretic velocity.

3. Cholesterol and quartz adsorb gelatin from dilute solution in the phosphate buffer. Red cells, on the other hand, even after 24 hours contact with gelatin solution, retain their previous velocity.

4. Pregnant and non-pregnant white female humans have the same red cell cataphoretic velocity. (The cells were not agglutinated.)

5. In a series of severe anemias no significant change in cataphoretic velocity was in general apparent, although marked changes in the morphology of the red cells were present.

I am indebted to Professor L. Michaelis for much valuable advice received in connection with this investigation.

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THE VISUAL ACUITY OF THE HONEY BEE.

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I.

Nature of Work.

1. The visual acuity of the human eye varies in a specific way with the illumination. At low intensities visual acuity is low; as the intensity increases, visual acuity increases with it, at first slowly, then more rapidly; and finally at very high illuminations further increase in intensity produces no augmentation of acuity (Uthoff, 1890; Koenig, 1897).

This extraordinary dependence of visual acuity on illumination has been known for many years. However, it has remained without explanation until we recently suggested a theory to account for it (Hecht, 1927-28). The idea underlying this explanation is as follows. Visual acuity measures the resolving power of the retina. The resolving power of a surface composed of discrete receiving elements varies inversely with the distance between the centers of the elements; or more generally, it varies with the number of elements present in a unit area. The retina is such a surface since it is made up of discrete rods and cones. The variations in visual acuity with illumination would therefore suppose that the number of visual elements per unit area of retina varies with the intensity. But since the number of rods and cones in the retina is fixed anatomically one must suppose it to vary functionally. To accomplish this, it has been assumed that the thresholds of the retinal elements are not the same, but are distributed in relation to the illumination in the usual manner of populations. Consequently the number of elements functional in a given area varies with the illumination; and when expressed quantitatively,

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this relation describes in detail the dependence of visual acuity on the intensity of illumination.

This work on visual acuity is confined to the eye of man. Its theoretical implications are many (Hecht, 1927-28) and involve consideration of the problems of intensity perception and intensity discrimination; it may even concern the matter of color reception and hue discrimination. The ideas underlying it should therefore be tested in a variety of ways. If the explanation of the dependence of visual acuity on illumination which we have proposed is generally valid; in other words if the number of receptor elements functional in a sense organ does vary with the intensity of the stimulating agent, then visual acuity should vary with the illumination in other animals in a manner resembling that in man.

There exist, however, no measurements of this nature with any other organisms. We have therefore studied the visual acuity of the arthropod eye, a sense organ which is built and is functional on a wholly different principle from the vertebrate eye (Exner, 1891).

2. The problem of studying the vision of animals other than man is made difficult by the impossibility of verbal communication between the animal and the experimenter. The best method of solving this difficulty up to the present has been by means of the conditioned reflex, used originally by Pavlov (1927) with mammals, and in a modified form by v. Frisch (1915) with bees. Though we have nothing but praise for this method and for many of the results achieved by it, we nevertheless tried to find a more direct one, which would involve no training of animals.

We started with the common observation that most animals with eyes respond to a sudden movement in their visual field, and in terms of the following considerations converted it into a method of measuring visual acuity quantitatively. If the visual field of a sensitive animal is made up of a pattern of dark and illuminated bars of equal size, the animal will respond to a displacement of this field only when it can distinguish the components of the pattern. In case the animal cannot resolve the black and white bars, the field will appear uniformly illuminated and displacement of the pattern will elicit no response. If visual acuity varies with illumination, then the capacity to respond to these movements in the visual field will depend on the illumination

and on the size of the pattern. One could in this way determine the relation between the size of the bars in the pattern and the minimum illumination at which it causes a response in the animal.

3. Our experiments were made with the honey bee because of its abundance and its genetic uniformity. It is sensitive to changes in its visual field, and responds by a reflex, sidewise movement of the head and thorax. If the bee is crawling, the response becomes evident by a sudden change in the direction of its progression, which is opposite in sign to the movement in its environment.

We prepared a series of plates composed of equally wide opaque and translucent bars, each plate having a different size of bar. Our experiments then consisted in determining for each size of pattern the minimum illumination at which a bee will just respond to a movement of that pattern. The reciprocal of the visual angle subtended by each size of bar is then the visual acuity of the eye at the corresponding illumination. The results show that the visual acuity in the honey bee varies with the illumination in a way which is similar to the human eye.

II.

Apparatus and Procedure.

1. The details of the apparatus which we used in these experiments can be made out with the help of Fig. 1. A source of light (1000 watt, concentrated-filament, mazda lamp) in dark room *B* illuminates a plate of opal glass in the wall of an adjacent dark room *A*, in which the experiments are performed. The opal plate is immediately behind an accurately controlled diaphragm (Fig. 1) the opening of which determines the area of the plate which radiates light into dark room *A*. This light is reflected upward by a mirror, and impinges on a piece of opal glass 25 x 15 cm. in size. Immediately upon this opal glass plate rests a piece of transparent glass of the same size, with the experimental opaque and transparent bars on it. These two pieces of glass,—the opal plate and the pattern plate,—fit into the opening in a frame which is moved by an attached knob, and which slides freely in a pair of grooves in a larger frame. The movement of the frame with the two plates constitutes the moving visual pattern.

The bee is confined in a glass compartment 10 x 10 cm. and 3 cm. high, held about a centimeter above the pattern. The bottom of the compartment is the plate on which the bee crawls. It is an ordinary photographic plate with the halide completely removed but the gelatine left so as to serve as a foothold for the crawling bee. These plates are used because they are uniform; they may be removed very frequently and washed and dried.

The bee's creeping compartment and the movable black and white stripes of the visual field are tilted at an angle of about 30° . Because of the bee's negative geotropism this tends to make the animal crawl upward in a straight line, so that

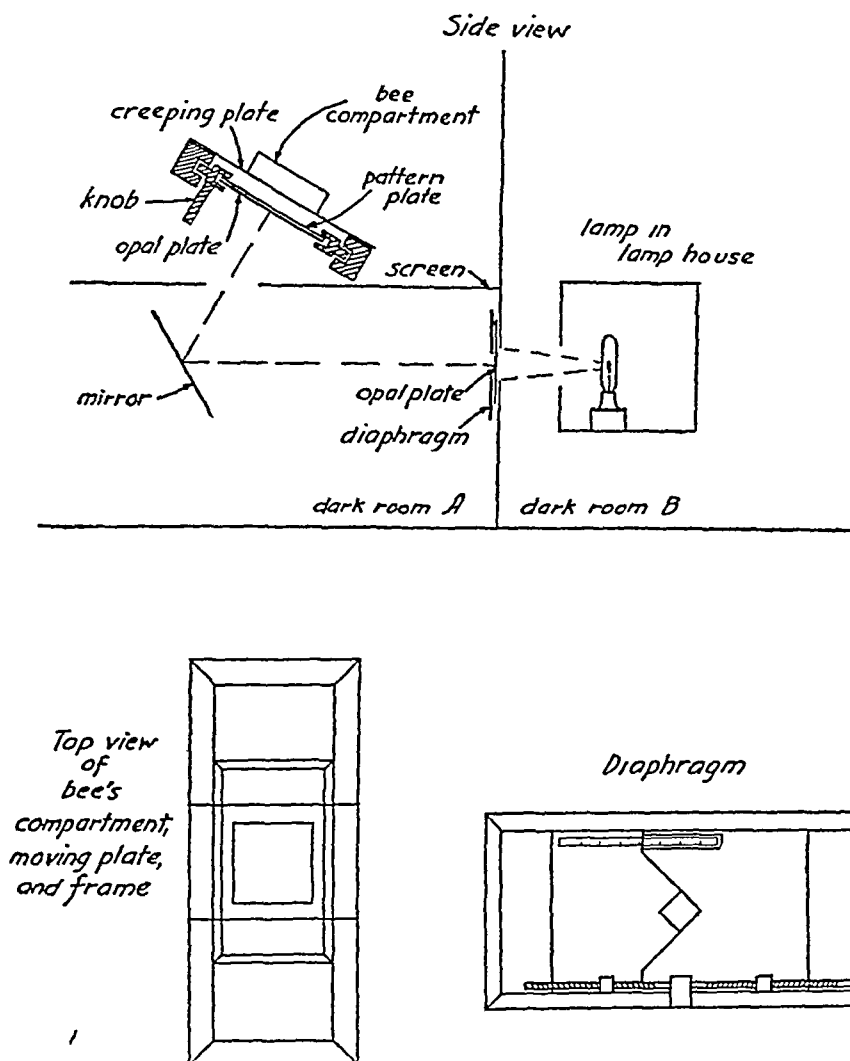


FIG. 1. Diagrammatic representation of apparatus for measuring the visual acuity of bees.

deviations from its linear progression due to the movement of the visual field are easily observable. A mirror placed above the bee's creeping compartment gives the observer a top view of the bee and of the visual field.

We used thirteen sizes of stripes. The larger ones we made by putting opaque

black paper between two pieces of glass. The finer ones were engraved on glass by Max Levy and Company of Philadelphia, and have the opaque areas filled with black printer's ink. The visual acuities furnished by these plates depend on their distance from the eye of the bee. On several occasions we measured this distance. It varies slightly from plate to plate and from bee to bee. The average is 17.3 mm. representing the distance from the center of the creeping bee's eye to the plate which has the pattern on it. In Table I is given the dimensions of the lines and spaces, the resulting visual angle, and the reciprocal of the visual angle representing the visual acuity. As in the case of ophthalmological practice a visual angle of 1 minute corresponds to a visual acuity of unity.

TABLE I.
Designations and Properties of Pattern Plates Used in the Experiments.
Distance of pattern from center of bee's eye is 17.3 mm.

Designation	Width of bar	Visual angle subtended by bar	Visual acuity $\times 10^4$
	mm.	min.	
A	19.0	2862.0	3.50
B	12.4	2136.0	4.68
C	9.4	1710.0	5.85
D	6.3	1200.0	8.33
E	4.7	912.0	10.96
F	3.2	630.0	15.87
G	1.27	252.0	39.68
H	0.847	168.0	59.52
I	0.635	126.0	79.37
J	0.508	100.8	99.21
K	0.423	84.0	119.0
L	0.363	72.0	138.9
M	0.318	63.0	158.7

The illumination of the striped pattern is varied in two ways. The 1000 watt lamp in dark room B may be placed in one of three fixed positions. Position I is 15 cm. from the opal plate in the wall between the two dark rooms; Position II is 50 cm.; Position III is 170 cm. from the opal plate. We made six separate calibrations of ten measurements each of the relative intensities emitted by the opal plate when illuminated by the lamp in each of the three positions. The relative intensities, putting Position I equal to 1 are:—Position II, 0.0862; Position III, 0.0140. The diaphragm controlling the area of the radiating opal plate gives a span of over 1:10. The smallest opening used is 2 cm. square; the largest, 8 cm. square. The diaphragm when used with the lamp in the three positions enables one to provide accurately and continuously an illumination covering a range of over 1:1000.

The size of the opening in the diaphragm can be read to 0.1 mm. by an engraved metal scale attached to one leaf in the diaphragm and a pointer wire attached to the other. We did not rely on the fact that the illumination on the striped pattern is proportional to the size of the radiating area in the diaphragm, but measured the actual brightness of the striped pattern when illuminated by eleven different areas of the diaphragm. The measured points, plotted as scale reading on the diaphragm against the logarithm of the resulting brightness, fall on a smooth curve. This we drew on a sufficiently large scale to use as a calibration curve.

2. The procedure in making the experiments is as follows. Active worker bees from our own colony are taken from in front of the hive. They are lightly anesthetised and the wings clipped. After recovery they are kept in wire cages, several to a cage, where they have access to plenty of honey. A number are then tested individually in the apparatus and from them are selected vigorous bees whose responses to the moving visual field are sharp. These are marked by differently colored spots of paint placed on the thorax or abdomen. They are kept warm, dry, and in the dark.

To make a measurement, a visual acuity plate is put into the movable frame, the light is turned on for a given illumination, and a bee is placed in the creeping compartment. The animal at once begins to creep and continues actively all the time it is in the compartment. First it creeps upward on the floor of the cell. Then it tries to crawl up the sides. This usually results in its dropping to the bottom, where it again crawls upward. If it can successfully creep up the side it may continue upside down on the top; in which case it arrives at the bottom and crawls up again on the floor of the compartment. Very often it drops from the roof, and starts its journey on the floor again. The fact that the floor is the only illuminated face of the compartment also helps to keep the bee creeping there. The result is that at very frequent intervals the animal creeps across the visual field. After it has moved about 2 cm. and its direction of progression is clear, the pattern plate is moved with a sharp but not too rapid motion. Depending on whether the bee responds or not, the intensity of the field is lowered or raised and the bee tested again. This is continued until the illumination is found at which the bee just responds to the movement.

It is an extraordinary sight to watch the precision with which a bee changes its direction of creeping under these conditions. If the pattern is moved, say, to the left, the creeping bee swings sharply to the right through an angle which is easily 45° and may be much more, and continues creeping in the new direction. During a single crawl of perhaps 10 cm. we have frequently made a bee alter its direction, right and left by moving the plate left and right, as many as four or five times in rapid succession.

In this connection we have made an observation often enough to be quite certain of its reality. When the plate is moved slowly, and the animal has established its new direction, if the plate is suddenly stopped the bee swings into a new direc-

tion as if the plate had suddenly been moved in the opposite direction. The appearance is reminiscent of the common nystagmus of vertebrates.

After the reading has been checked the bee is removed, placed in the cage in the dark, and another bee is tested in the same way. This is usually done with about ten bees. After all of them have been run through with one size of stripes, another plate is placed in the movable frame and the same bees tested on a different size of stripes. This procedure is continued at regular intervals during the day, in the course of which six or seven different sets of stripes are tested. By choosing the proper plates one can in this way cover very nearly the whole range of visual acuity with each bee. The next day, with a fresh batch of bees one can then investigate the whole range again, but with visual acuities in between those already tested. We have found this better than to test each bee to all the plates in one day, because the animals seem to respond more vigorously and survive better if they are tested at intervals of about an hour.

III.

Experimental Results.

1. The preliminary work for the experiments occupied over six months of almost daily effort before any of the measurements to be reported here were made. Beginning with the fact that bees respond to a movement in their immediate environment, we tried to elicit this response under controlled conditions which would make measurements possible. For nearly three months all kinds of devices for producing a controllable movement to which the animal would give a fixed response were tried without success, until the tilted compartment with the moving stripes below it solved the problem. The angle of tilt is quite important and is a compromise between a steep angle which causes a pronounced geotropic creeping and a gentle slope which keeps the bees from rolling down the incline. The gelatine covered creeping plate helps to increase the angle of tilt. The rest of the preliminary experiments were concerned with designing and testing different types of apparatus and different means of producing the visual pattern, with the results already indicated.

After all the conditions had been properly set, we began the final measurements early in June 1928. We made measurements with 91 normal animals in 9 days. The data are given in Table II where every measurement with every animal is recorded. The logarithms of the intensities are given instead of the intensities themselves for reasons which will become evident in a moment.

[illegible]

* In Column E the values to the left were made with the lamp in Position III; the values to the right with the lamp in Position II. In Column K, the left values are with the lamp in Position II, the right values in Position I.

TABLE II.—Continued.

Date	Bee No.	Logarithm of illumination for visual acuity												
		A	B	C	D	E*	F	G	H	I	J	K*	L	M
June 15, 1928	55		2.762						1.945		0.146		0.534	1.140
	56		2.527						1.817		0.086		0.378	
	57										0.107		0.738	1.388
	58		2.623						1.955		0.071		0.660	1.218
June 16, 1928	59	2.373		1.060			1.452	1.768		0.058				1.080
	60	2.008		2.713			1.610	1.710		0.022				1.145
	61	2.350		2.713			1.296	1.858		0.080				1.230
	62	2.206		2.963			1.402							1.310
	63	2.420		2.940			1.386	1.777		0.008				1.245
	64	2.465		2.976			1.300	1.946		0.090				1.124
	65	2.382		2.879			1.545	1.435		1.885				0.930
	66	2.455		2.870			1.509	1.798		1.963				0.902
	67	2.587		2.623			1.170	1.722		0.016				1.185
	68	2.587		2.920			1.495	1.790		1.828				1.388
June 18, 1928	69	2.883		2.868			1.380	1.817		1.970				1.100
	70	2.018		2.730			1.368	1.828		1.980				1.167
	71	2.161	2.505		1.150				1.930		0.092		0.534	
	72	3.974	2.623		2.987				1.768		1.980		0.406	
	73	2.135	2.324		1.132				1.885		0.100		0.345	
	74	2.465	2.299		1.301				1.990		0.020		0.482	
	75	2.527	2.685		2.904				1.965		0.080		0.425	
	76	2.373	2.825		1.150				1.970		0.208		0.716	
	77	2.190	2.713		1.060				1.930		0.100		0.258	
	78	3.885	2.838		1.234				1.828		0.035		0.534	

June 19, 1928	79	2.420	2.623	1.210			1.880	0.071		0.444
	80	2.161	2.495	1.069			1.850			0.534
	81	3.820	2.642	2.890			1.734	1.904		0.550
	82		2.746	1.176			1.888	0.139		0.639
	83	2.219	2.778	1.301			1.912	0.100		0.500
	84				1.578	1.886	1.980		0.192	1.222
	85				1.358	1.790	0.004		0.345	1.326
	86				1.195	1.750	1.946		0.444	1.199
	87				1.365	1.798	1.970		0.118	1.109
	88				1.262	1.695	1.955		0.236	1.180
	89					1.849	0.080		0.406	1.026
	90				1.419	1.817	1.895		0.303	1.124
	91				1.098	1.828	0.100		0.236	1.230
Average.....		2.300	2.639	1.051	1.294	1.422	1.889	0.093	0.257	0.521
							1.994			1.177

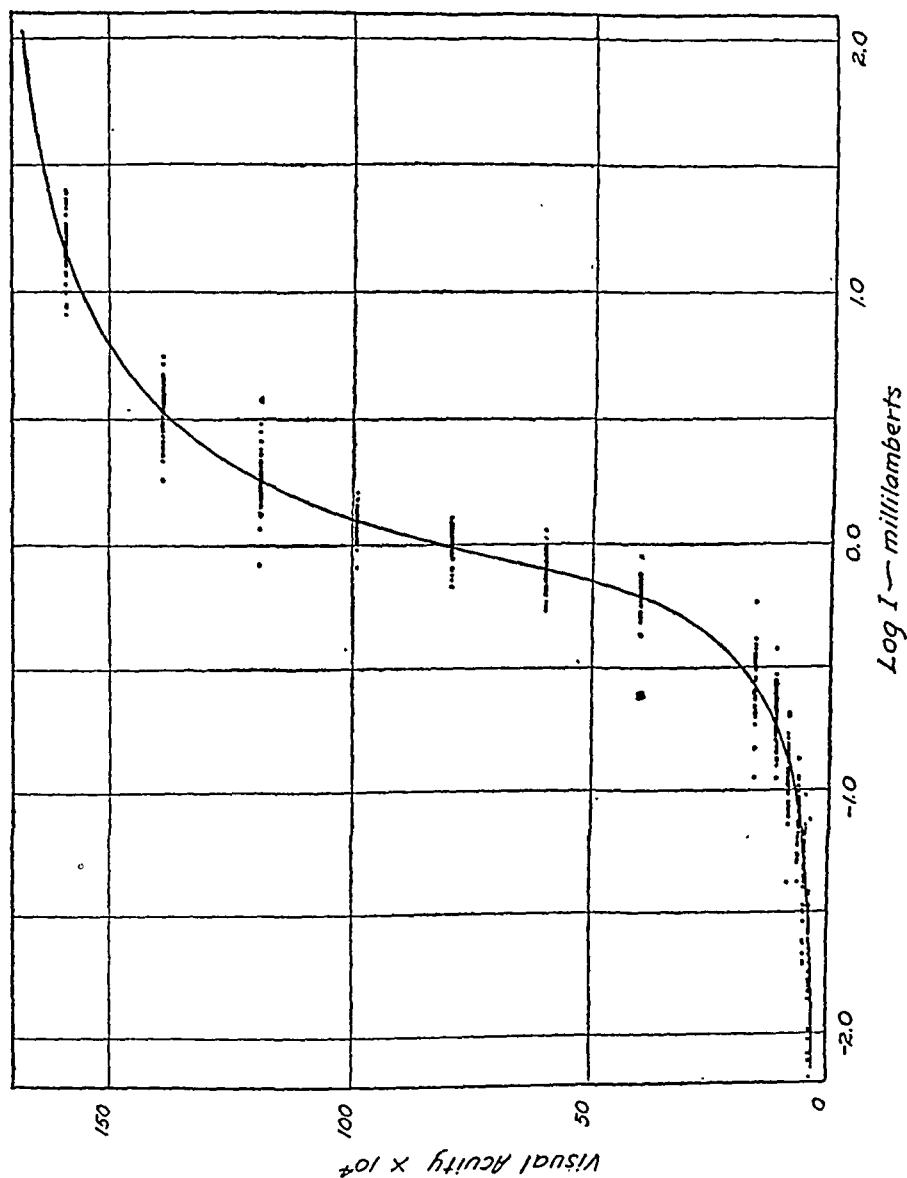


FIG. 2. Relation between visual acuity and illumination. An effort has been made to represent with a dot each measurement with each bee. This is manifestly impossible in the places where the measurements come very close together. The plot therefore shows the general way in which the measurements run. The full details are given in Table II. The curve is drawn through the mean values of the measurements.

2. The way in which in Table II the different individuals vary in the minimum intensity for a given visual acuity is significant. The range of variation is greatest at low visual acuities; it decreases as the visual acuity increases; and at the highest visual acuities, it again becomes larger. In Fig. 2 the same data are presented graphically. At once the reason for the curious distribution of the variation becomes clear.

The relation between visual acuity and the logarithm of the illumination is sigmoid, much as for the human eye. It follows that at low visual acuities a very small difference in visual acuity corresponds to a large difference in intensity; at the middle range, visual acuity is almost directly proportional to $\log I$, the curve being quite steep; finally at high visual acuities a condition prevails similar to that at low acuities. At the two ends a very slight individual difference in the animal shows itself in a large difference in minimum illumination, whereas the reverse is true in the middle ranges. This is shown by the closeness with which the individual points at the lower end adhere to the curve drawn through the average values of the data.

3. The shape of the curve in Fig. 2 indicates that the experiments cover the whole range of visual acuity of which the bee is capable. The maximum value lies between 0.016 and 0.017. This is below the visual acuity of the human eye at the lowest perceptible illuminations. Our maximum visual acuity is about 1.5; our minimum is about 0.03 (Koenig, 1897; Hecht, 1927-28). Incredible as it may seem, the bee's greatest capacity for the optical resolution of its environment is never better than ours is at our worst.

The disparity in the visual acuity of our eyes and those of the bee is even greater than this and is brought out by considering the visual acuities at the same illuminations. The maximum visual acuity in both cases occurs at very nearly the same intensities of illumination and corresponds to a brightness of between 50 and 100 millilamberts. Our maximum here is about 1.5; the bee's maximum is 0.017. In other words we can resolve the environment about 100 times better than a bee can. That this low value is no laboratory product is borne out by the experiments of Baumgärtner (1928) in which bees on the wing in the field were shown to have a similar, almost negligible, form discrimination. Baumgärtner, using v. Frisch's conditioned-reflex

method, found that in broad daylight a bee can just recognize a 2 cm. square at a distance of about 40 cm. This corresponds to a visual angle of a little less than 3° , and a visual acuity of about 0.006. This is even lower than our values, but still of the same order of magnitude.

4. The intensity range over which the bee and the human eye function in relation to visual acuity is such that for each the maximum visual acuity is about 50 times the minimum. In both cases the maximum occurs at about the same intensity of illumination, 100 ml. The lowest visual acuity for the bee is 0.00035 and occurs at 0.020 ml. At intensities below this the animals wander all over the creeping compartment and do not respond to the movements of even larger patterns than here recorded. This is an intensity range of about 3.5 logarithmic units. The same span of visual acuity is spread by our eye over a range of over 7 logarithmic units of which 2.5 are controlled by the rods and 4.5 by the cones. Our eyes thus function over intensities 3.5 log units lower than those of the bee.

IV.

Anatomical Basis of Visual Acuity.

1. In spite of these differences in magnitude and range between the visual acuity of the human eye and that of the bee's eye, the relation between visual acuity and illumination is essentially the same in the two. The resolving power of the bee's eye is low at low illuminations and increases with the logarithm of the illumination up to a maximum, in the same S-shaped way as the human eye. The same kind of theoretical treatment is therefore indicated.

Differences in resolving power such as are found here mean differences in the distances which separate the centers of the receiving elements. The data, then, if interpreted naïvely would require that the ommatidia vary in their distance apart, assuming different positions depending on the intensity. This obviously cannot be true because the eye of the bee is a fixed organ and does not change either its shape or its size under illumination. The data must therefore be interpreted in some other way, the requirement being to secure a functional separation of ommatidia which are structurally fixed.

To solve this problem it is supposed that the receptor elements in the ocular mosaic do not all possess the same threshold, but that the threshold varies among the ommatidia as does any other characteristic in a population. This then works out so that at low illuminations only a few ommatidia are functional. Since these are distributed at random, they will be far apart and will give the same result functionally as if there were no receiving structures between them. As the illumination increases, more and more ommatidia become functional; the distance between functional elements becomes smaller; and the resolving power becomes greater. This continues until an illumination is reached which is above the threshold of the most insensitive ommatidium. All the elements will then be functional, and no further increase in visual acuity can take place.

It is apparent that such an explanation is in general agreement with the data, and could be formulated in strictly quantitative terms for comparison with the data. However, before this can be done in a significant manner it is necessary to examine more closely the nature of the data in their relation to the structure of the eye.

2. In the experiments the animals were measured as they crawled in directions nearly parallel to the length of the stripes. The visual pattern is therefore registered across the long axis of the eye. Let us neglect for a moment the fact that since the plane of the pattern is roughly perpendicular to the long axis of the eye, the successive bars in the pattern will occupy a decreasingly smaller visual angle. At an illumination when all the elements are functional, the maximum visual acuity will then occur when a horizontal row of elements receives light, and an adjacent row receives no light, and so on. The size of the smallest perceptible pattern will correspond to the visual angle which separates the centers of two adjacent elements.

If the elements were all the same angular size and were evenly distributed on the surface of the eye, the maximum visual acuity would be perceptible all over the eye. But the elements in the bee's eye are not uniform in angular dimension. Then the maximum visual acuity can obviously be perceptible only at that point on the eye where the population density of visual elements is at a maximum, and the resulting angular separation a minimum. In the very nature of our measurements the visual acuity corresponding to any illumination is

always the maximum visual acuity at that intensity. Therefore at any illumination, no matter how many functional elements it represents, the visual acuity, as given, is determined at that area on the eye where the population density of functional elements is greatest. This is a fundamental concept in understanding the data. Fortunately it may be tested experimentally.

TABLE III.

Angle Subtended by Walls of Ommatidia in Longitudinal Section.

Ommatidial density in different parts of eye. Data obtained graphically from Abb. 29 in Baumgärtner (1928).

Ommatidium No.	Angle	Ommatidia per unit angle
	<i>degrees</i>	
5	2.22	0.45
10	1.89	0.53
20	1.56	0.64
30	1.41	0.71
35	1.33	0.75
40	1.22	0.81
45	1.13	0.89
50	1.02	0.98
55	0.94	1.07
60	0.88	1.13
65	0.88	1.13
70	0.92	1.09
80	1.02	0.98
90	1.10	0.91
100	1.19	0.84
110	1.32	0.76
120	1.52	0.66
130	1.97	0.51
140	3.57	0.28

The measurements as plotted in Fig. 2 show that the maximum visual acuity of which the bee is capable at the highest illuminations is very nearly 0.017. This corresponds to a visual angle of 0.98°. Since at these illuminations all the ommatidia are functional, this experimentally determined, minimal, angular separation should correspond to the smallest vertical separation between adjacent ommatidia as determined anatomically. Baumgärtner (1928) has

recently made a histological study of the bee's eye, and has measured the angular separation of adjacent ommatidia in different sections of the eye. In vertical section the angular separation increases about four times from center to periphery. The smallest separation is near the middle, in the lower half of the eye and includes between 20 and 25 elements in vertical section. Its value lies between 0.9° and

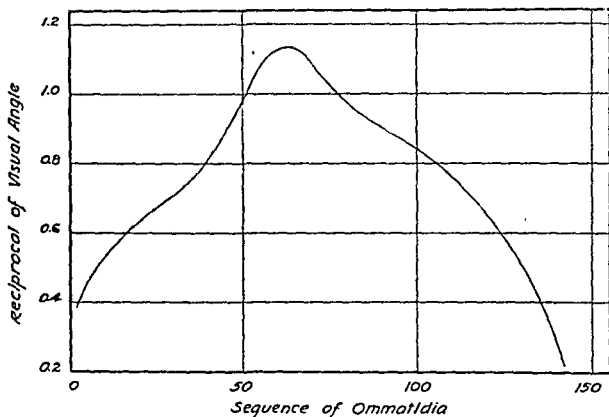


FIG. 3. Morphological distribution of visual acuity in a longitudinal section of the bee's eye. Data taken from Abb. 29 in Baumgärtner's (1928) paper. The abscissas are the ommatidia in sequence beginning with the ventral ommatidium and going dorsally. The ordinates are the reciprocals of the angle made by the walls of the corresponding ommatidia. The ordinates are equivalent to the relative number of ommatidia in a unit angle in different parts of the eye.

1.0° . This agreement between experimental and morphological findings indicates that visual acuity is determined by the region of maximum population density.

3. The evidence for this conclusion becomes even better when the morphological findings are examined in detail. Baumgärtner measured the angular separation of the two walls of each ommatidium in the longitudinal section of the eye. Table III contains the data,

taken graphically from Fig. 29 of Baumgärtner's paper, which describe his findings with 8 such sections. The data are plotted in Fig. 3. Here the abscissas are the ommatidia numbered in serial order beginning at the bottom of the eye; the ordinates are the reciprocals of

TABLE IV.

Relation Between Visual Acuity and Illumination in Bees With the Central Area of the Eye Painted Out.

Non-functional area is one-quarter of normal. Illumination in millilamberts; visual acuities as in Table I.

Date	Animal No.	Logarithm of illumination for visual acuity					
		F	G	H	I	J	K
June 28, 1928	1	1.493	1.948	0.092	0.165	0.610	
	2	1.666	1.942	0.016	0.127	0.406	1.222
	3	1.558	1.919	1.960	0.254	0.345	1.283
	4		1.817	1.988	0.195		
	5	1.613	1.673	1.983	0.202	0.693	
	6	1.502	1.900	0.028	0.242	0.603	1.128
	7	1.593	1.984	0.058	0.213	0.534	1.065
	8	1.423	1.774	1.959	0.120	0.438	1.088
	9	1.240	1.793	0.060	0.240	0.364	
	10	1.704	0.006	0.105	0.192	0.479	1.339
	11	1.602	1.890	0.040	0.170	0.450	1.185
	12	1.550	1.877	0.024	0.146	0.201	1.300
June 29, 1928	13		1.801	0.010	0.172	0.359	1.153
	14		1.946	0.038	0.195	0.550	1.205
	15		1.867	0.030	0.152	0.503	
	16		1.990	0.111	0.215	0.610	1.121
	17		1.921	0.072	0.159	0.163	1.236
	18		1.928	0.090	0.195		
	19		1.817	1.959	0.133	0.367	1.321
	20		1.885	0.004	0.150	0.463	1.191
	21		1.863	1.988	0.127	0.118	
Averages.....		1.540	1.883	0.029	0.179	0.435	1.203

the angular separation of adjacent ommatidia, and therefore represent the density of ommatidia distribution in vertical section. Attention is called particularly to the region of great ommatidia density in the lower half of the eye where occur the elements of smallest angular separation already referred to. The arrangement is not unlike the

concentration of the smallest retinal elements which form the fovea of mammals.

What should happen if this region of high ommatidial density were rendered functionless? Clearly, the maximum visual acuity should be decreased, since it would have to be mediated by the region of the eye with a lower concentration of visual elements. Moreover the whole relation between visual acuity and illumination should become depressed, in that the visual acuity at any illumination should be lower than normal and dependent on the extent of the fovea which has been rendered nonfunctional.

TABLE V.

Comparison Between Visual Acuity of Normal Bees and of Bees With Central Part of Eye Painted Out.

Logarithm of illumination	Visual acuity $\times 10^4$		Painted Normal
	Normal	Painted	
1.540	20.6	15.9	0.77
1.883	58.3	39.7	0.68
0.029	87.2	59.5	0.68
0.179	110.4	79.4	0.72
0.435	133.9	99.2	0.74
1.203	159.0	119.0	0.75

We made experiments in which a spot of black paint was placed in the center of each eye. It is, of course, difficult to place such a patch of paint with very great accuracy, and to do it uniformly from eye to eye. We tried to cover about a quarter of the area of the eye. In vertical section such a patch extends over about half the ommatidia and eliminates those elements falling between ommatidium No. 35 and No. 100. With the fovea removed down to about ommatidium No. 35, the maximum density of elements is between 0.75 and 0.80, which corresponds to an angular separation of 1.3° . Since the minimum angular separation for the normal bee is very nearly 0.9° , the maximum visual acuity of the bees with the fovea painted out should be between three-quarters and two-thirds of normal at the highest illuminations. Moreover, since, as is apparent from Fig. 3, the ommatidial density decreases in a roughly uniform manner from the center

to the periphery, the visual acuities at any other illuminations should be correspondingly lower.

We made two series of measurements in the usual way in which the relation was determined between visual acuity and illumination for

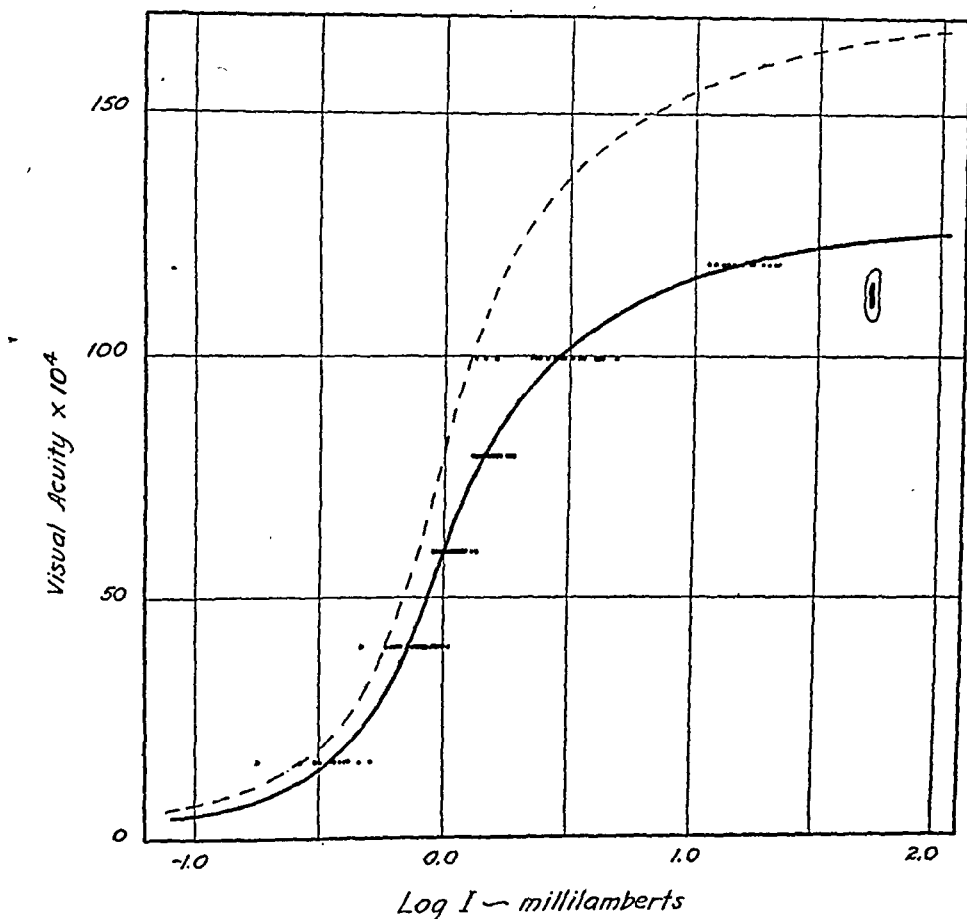


FIG. 4. Relation between visual acuity and illumination for bees with the central part of the eye painted out as shown in the figure. The points are individual measurements. The broken curve is the normal relation taken from Fig. 2. The full curve is made from the normal curve by multiplying its ordinates by 0.75.

bees with a central spot of paint on each eye. One series contained 12 animals; the other 9 animals. The results are given in Table IV in which the individual measurements are recorded as before. Table V

gives the average values of the intensities, the corresponding visual acuities for the painted bees, and for normal bees as taken from Fig. 2. The last column gives the ratios of the visual acuity of the painted eye to that of the normal eye at the same intensity. The ratio is obviously constant.

The data are also presented in Fig. 4, in which the two series are not treated separately. The broken curve in Fig. 4 is the normal curve taken from Fig. 2. The solid curve through the points is this same normal curve with its ordinates multiplied by 0.75. This agreement with expectation is surprisingly good, and shows that visual acuity determination in the bee's eye is a regional function, and depends on the utilization of the part of the eye containing the densest population of active elements.

It is significant that the region of densest ommatidia population is in the lower half of the eye, because the lower half of the eye is concerned with our measurements. This is apparent from the relative positions of the eye and the pattern. However, we tested it experimentally as well by painting out the upper half of the eye. Such a procedure seems to have no effect on the general behavior of the bee nor on its visual acuity.

4. Our measurements of visual acuity were so conducted that the pattern was registered on the long dimension of the eye. Consider an illumination when only a fraction, say one-ninth, of the visual elements are functional. Since the thresholds are distributed at random, any two functional elements are separated by two non-functional ones. Assuming for the moment that the mosaic is uniform, the angular distance between the functional elements will now be three times as great as in the fully functional eye. If the visual pattern varied in many directions, the resulting visual acuity would then be a third of its maximum value. But the present pattern is a uni-directional one, and can be resolved only along the vertical axis of the eye. The visual acuity corresponding to the present situation will then be determined by the projection on the long axis of the visual angle between any two functional elements. Moreover the ocular mosaic is not uniform. The visual acuity will then be determined by the apparent vertical separation of functional ommatidia in the region of greatest density.

This region of maximum density is always small, as Fig. 3 shows, and will contain only a few functional elements for which this vertical separation corresponds to the maximum visual acuity at that illumination. Undoubtedly a certain minimal number of such elements are required for the reception of the stimulus. Since the eye is very

TABLE VI.

Relation Between Visual Acuity and Illumination for Animals With One-half the Eye Painted Out.

Illumination in millilamberts; visual acuity as in Table I.

Date	Animal No.	Logarithm of illumination for visual acuity					
		E	F	G	H	I	J
June 23, 1928	1	1.384	1.384	1.734	0.170	0.444	
	2	1.578	1.716	0.071	0.184	0.679	1.018
	3	1.563	1.710	0.032	0.225	0.518	1.034
	4	1.614	1.675	0.050	0.213	0.695	1.178
	5	1.314	1.434	1.778	0.061	0.574	1.310
	6	1.519	1.660	0.076	0.206	0.580	0.863
	7	1.477	1.549	0.028	0.282	0.518	1.072
	8	1.463	1.675	1.930	0.231	0.667	
	9	1.661	1.777	0.001	0.170	0.664	1.193
	10	1.608	1.675	1.965	0.050	0.464	1.201
	11	1.402	1.648	1.939			
June 25, 1928	12	1.419	1.608	0.050	0.208	0.325	1.018
	13	1.470	1.614	1.817	1.970	0.438	1.218
	14	1.343	1.777	1.863	0.138	0.500	1.172
	15	1.545	1.552	1.969	0.120	0.708	1.026
	16	1.533	1.638	1.828	0.220	0.345	
	17	1.402	1.722	0.004	0.246	0.514	1.153
	18			1.963		0.565	
	19	1.384	1.648	1.912	0.110	0.530	
Averages.....		1.482	1.637	1.948	0.165	0.540	1.112

nearly symmetrical in its two halves, there will be a symmetrical distribution of these few critical elements on either side of the central vertical axis. If now one side of the eye were rendered functionless, this should at once reduce the number of densely grouped elements by half. To get the same number of functional elements as before in order to resolve the pattern, elements which are more widely separated

would have to be drawn on, and at once the visual acuity should be decreased.

We made two series of experiments which test this; one with 11 bees and the other with 8 bees. In these we painted out approximately half of each eye, the dividing line being along the vertical axis. The results are given in Tables VI and VII, the latter containing the ratios of the visual acuities here obtained with those found for the normal bee at the same illumination. The ratios are seen to be nearly the same. This is brought out by Fig. 5 where the data are presented graphically. Through the experimental points there is drawn a con-

TABLE VII.

Comparison Between Visual Acuity of Normal Bees and of Bees With Anterior Half of Eyes Painted Out.

Logarithm of Illumination	Visual acuity $\times 10^4$		$\frac{\text{Painted}}{\text{Normal}}$
	Normal	Painted	
1.482	17.8	11.0	0.62
1.637	26.4	15.9	0.60
1.948	70.9	39.7	0.56
0.165	108.6	59.5	0.55
0.540	140.1	79.4	0.57
1.112	157.9	99.2	0.63

tinuous curve whose ordinates are 0.62 of the values for the normal, unpainted eye.

It is therefore apparent that the reduction of visual acuity as the result of painting out half the eye is of the kind which should occur if the resolution of the visual pattern is determined by the vertical separation of the functional ommatidia in the region of maximum density.

We made several series of experiments in which the functional part of the eye was further reduced. As is to be expected, the resulting visual acuity function becomes more and more depressed. The data are not given, because they are of no quantitative significance, since the actual part of the eye which remains unpainted is not definable in any quantitative terms as it decreases in size.

5. The conclusion to which we have just arrived, as well as the

facts which have led to it result in a minor paradox. In computing in Table I the visual angle corresponding to each of the pattern plates we considered the angle subtended by the two edges of a bar, one side

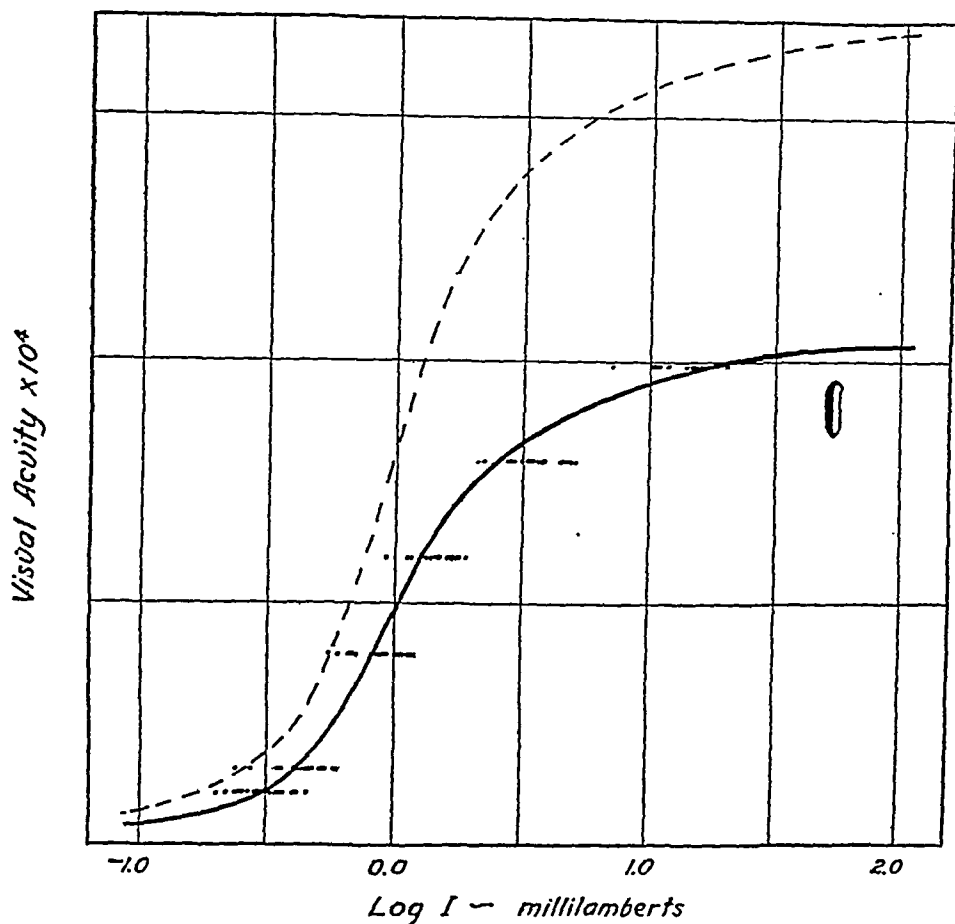


FIG. 5. Relation between visual acuity and illumination for bees with the anterior half of the eyes painted out. The points are individual measurements. The broken curve is the normal curve of Fig. 2. The full curve is constructed from the normal by multiplying its ordinates by 0.62.

of the angle being the perpendicular from the center of the eye to the plate. The tangent of the visual angle is the width of the bar divided by the perpendicular distance of the eye from the plate.

If the plane of the plate were parallel to the long axis of the eye this treatment would correctly give the visual angle subtended at any part

of the eye. But since the plate is practically perpendicular to the long axis of the eye the visual angle subtended by a bar varies with the part of the eye which is concerned with the resolution of the pattern. The paradox then is that we have been comparing a visual angle measured on the plate at the perpendicular from the eye with an angular separation of ommatidia situated one-third of the way up the eye.

The paradox largely disappears when it is remembered that the head of the bee as it creeps is not rigid. Even at rest the head is not kept perpendicular to the creeping plane; and when the animal creeps the position and angle of tilt of its head vary considerably. For a good part of the time during creeping the visual angles as given in Table I probably represent the real angles. This is borne out by the coincidence between the anatomical visual angle and the experimental visual angle at the highest illuminations. This can hardly be considered fortuitous, especially when it is reinforced by the agreement of the experimental findings with those calculated on the basis of painting out portions of the eye. We have therefore retained the visual angles for the plates as given in Table I on the supposition that they are an appropriate designation of the resolving power of the eye at the intensities to which they apply

v.

Visual Acuity and Number of Elements.

1. Having considered the anatomical and structural basis of visual acuity in the bee's eye, we may now apply this information to a closer analysis of the relation between visual acuity and illumination as it concerns the number of elements functional,—an analysis postponed from the previous section. Off hand it would seem that, since visual acuity is determined by the vertical distance between elements in the region of maximum ommatidial density, the curve in Fig. 2 represents the number of functional elements in the vertical axis of the fovea corresponding to any illumination. This would be true if the ocular mosaic were uniform. Since however, the angular separation between adjacent ommatidia is not constant, the precise way in which this

varies must be considered in the conversion of visual acuity into number of ommatidia functional.

In Fig. 6 we have plotted part of the lower half of the bee's eye, ommatidium for ommatidium, beginning with ommatidium No. 25 and ending with No. 70 which is just in the center of the eye. The linear distance is in terms of ommatidial angle as taken from Baumgärtner's average curve given in Table III and in Fig. 3. This region covers a total angle of about 50° and therefore includes the whole range of visual angle encountered in the measurements.

The ommatidia lying in the center of the foveal region, that is Nos. 56 to 70, have practically the same visual angle, No. 56 has an angle of 0.92° ; those in between gradually decrease to No. 63 whose angle is

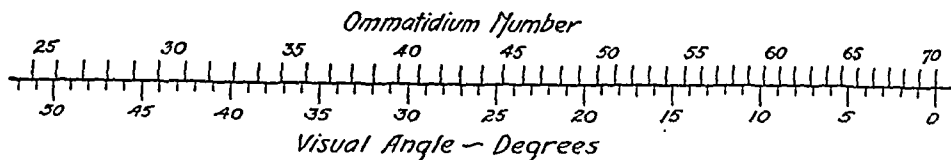


FIG. 6. Graphic representation of the method of finding the number of ommatidia included in a given visual angle. The lower scale is a linear scale of visual angles. The upper scale gives the angle subtended by the individual ommatidia, beginning with No. 70 at zero angle.

0.88° and then increase again to No. 70 which also has an angle of 0.92° . This variation of about 5 per cent may be neglected in view of the almost 100 per cent increase in visual angle shown by ommatidium No. 25. The significance of this middle region is that since it is the area of maximum population density the chance is greatest of its being the location of the most sensitive ommatidium. Therefore even at the lowest illumination, one functional element is most likely to be found here, and will continue to be here at all intensities. It may therefore be taken as the reference point from which to measure the vertical angular separation on the eye corresponding to any visual angle.

Which of the dozen or so elements in this practically uniform region of maximum density is the most sensitive one it is not possible to say. We have selected ommatidium No. 70, because the distance from it to any point on the lower half of the eye will always include the greatest

number of elements. It may be added that for the present purposes it matters only slightly whether we choose ommatidium No. 63 which is in the exact center of the fovea, or No. 70. The numerical results which we are after differ only insignificantly in the two circumstances and the principle is the same.

Below the diagram of the ommatidia in Fig. 6, we have drawn a simple linear scale of visual angle, beginning with zero at the upper edge of ommatidium No. 70. Using these two scales, one can read off how many ommatidia in the vertical projection on the upper scale

TABLE VIII.

Relation Between Visual Angle, the Number of Ommatidia Included in the Angle, and the Number of Ommatidia Functional per Unit Angle.

Visual angle	Number of ommatidia included	Number of ommatidia functional in unit angle
<i>degrees</i>		
47.7	43.55	1.00
35.6	34.83	1.25
28.5	29.24	1.49
20.0	21.67	2.01
15.2	16.84	2.59
10.5	11.78	3.70
4.20	4.65	9.37
2.80	3.08	14.14
2.10	2.30	18.93
1.68	1.84	23.67
1.40	1.53	28.46
1.20	1.31	33.24
1.05	1.14	38.20

are included in a given visual angle on the lower scale. At the greatest visual angle of 47.7° there are included 43.55 ommatidia. This means that the angle formed at the center of the eye by the lines from the two functional ommatidia nearest together includes 43.55 ommatidia in its projection on the longitudinal meridian of the eye. It therefore follows that only 1 out of 43.55 ommatidia is functional. This of course is only in projection on the vertical meridian, and does not mean the number of ommatidia which actually separate the two functional ommatidia in a direct line connecting them on the surface of the eye. This real value is naturally a whole number, and not a

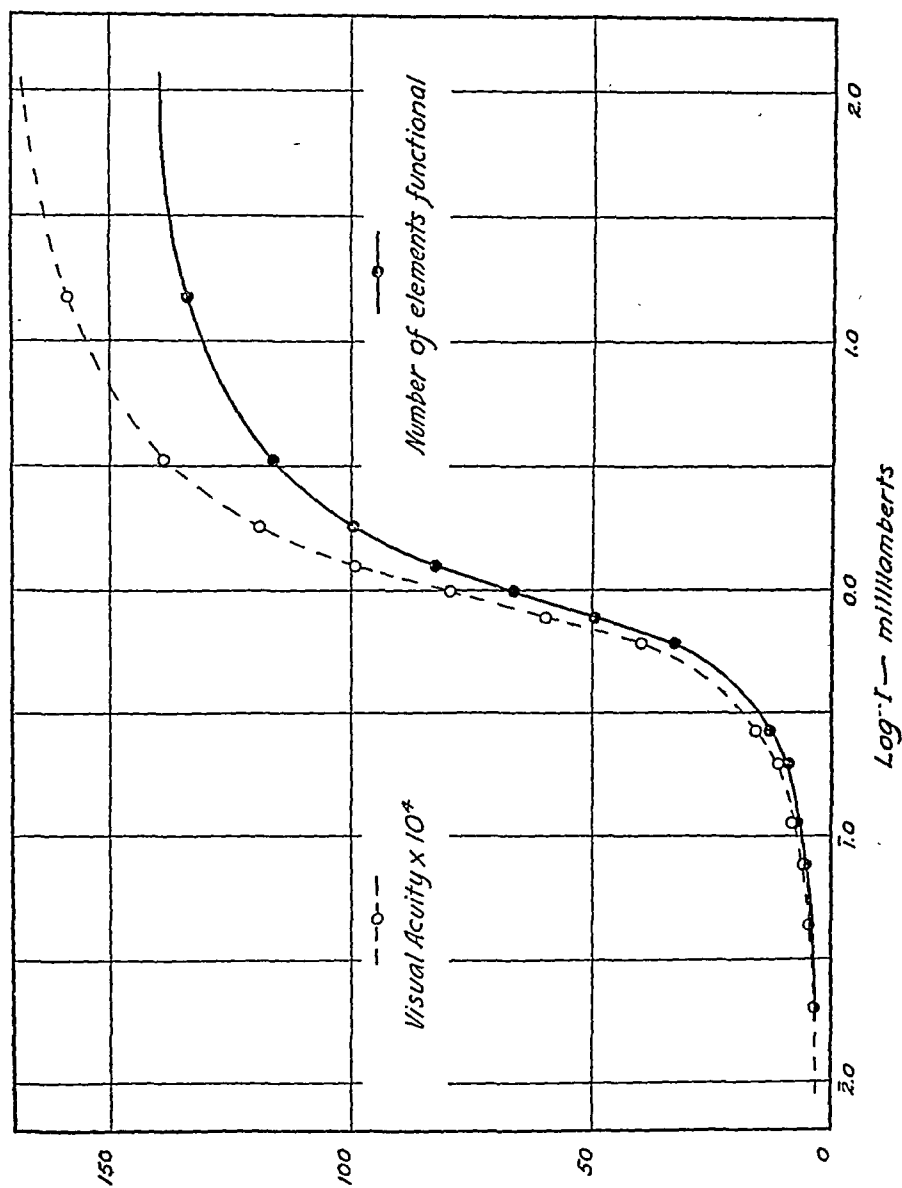


FIG. 7. Comparison between visual acuity and number of ommatidia functional in a given angular distance in their relation to the logarithm of the illumination. The relation between the number functional and $\log I$ is in the nature of an integral probability or distribution curve.

fractional one. The same procedure may be continued in order to determine the projected separation of the two functional ommatidia corresponding to any other visual angle.

The use of Fig. 6 in determining ommatidial density along the eye is a graphic method. Actually we determined these values arithmetically using exactly the same principle as described graphically. The values so secured are given in the second column of Table VIII. At a visual angle of 47.7° , there is functional 1 out of every 43.55 ommatidia. At a visual angle of 35.6° this will be 1 out of every 34.83

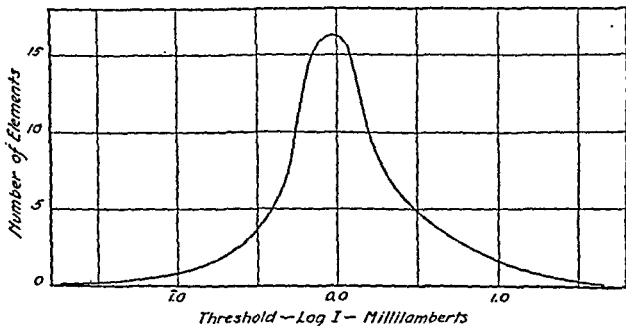


FIG. 8. Distribution of thresholds of the ommatidia in the bee's eye. The curve is the first differential of the number curve in Fig. 7, and is a differential probability or distribution curve.

ommata; or 1.25 out of every 43.55 ommatidia, if one considers the largest visual angle as the unit inside of which all the other angles must fall. In the same way all the other angles are computed with the results given in Column 3 of Table VIII.

We have thus made, in terms of the structure of the eye, a transformation from visual acuity data to the corresponding number of elements functional in a given distance on the vertical meridian of the eye. It is true that we do not have the real numbers but only the numbers in vertical projection. This is not without significance, as will be apparent presently. However, since the surface of the eye

is a hexagonal mosaic the real numbers are a simple geometric function of the numbers projected on the vertical meridian. The ratio of real distance to projected distance in such a mosaic is as $\sqrt{3}$ is to 1; so that any conclusions drawn for one holds for the other.

2. In order to show how these values of the number of functional elements are related to the visual acuity data from which they are derived, we have plotted both in Fig. 7. The upper curve is the visual acuity relation, the points being the averages from Table II. The lower curve is the number curve. So that the two might more readily be compared we have multiplied the ordinates of the number curve by 3.5; the ordinate units are thus the same in the two cases, and the lowest points are identical in the two. It is apparent that the number curve is lower, and more symmetrical than the visual acuity curve.

The number curve in Fig. 7 resembles the usual integral distribution curves of the statisticians, even as its first differential, the threshold curve, in Fig. 8 resembles the more commonly encountered differential distribution curves. These are indeed what they should be according to our idea of the relation between illumination, number of elements functional, and the resulting visual acuity. Therefore we may make our hypothesis quantitatively specific by stating it as follows. Taking the structural relations of the ocular mosaic as given by Baumgärtner (Table III), the data relating visual acuity and illumination (Table II) may be described with complete fidelity by assuming a distribution of the thresholds of the various ommatidia corresponding to the population curve of Fig. 8.

3. This distribution curve may be interpreted in two ways. So far we have tacitly assumed for the human eye and for the bee's eye that a given threshold is a permanent characteristic of a given element. The curve in Fig. 8 then represents the distribution of this characteristic in the population of ocular elements. For the human eye we even suggested a possible photochemical basis for this distribution (Hecht, 1927-28). However, one may conceive this situation purely in terms of probability. Assume that the threshold of a given element is not fixed, but can vary over the whole range included in Fig. 8. Then at any moment the probable distribution of elements over the entire threshold range is given by the curve in Fig. 8. This may therefore be used as the basis for visual acuity in precisely the same way as before.

We frankly incline toward the fixed threshold interpretation largely because it seems unlikely to us that a given cell can vary in its threshold from moment to moment over such a very large range of illuminations. However the matter may be tested experimentally by studying the dark adaptation of very small retinal areas in the human eye. If the increase in sensibility during dark adaptation for minute areas is as smooth and regular as for large areas then the threshold of a given element is most likely a permanent property.

It is apparent that the computations, experiments, and reasoning in Section IV and in a good part of the present section could have been avoided if we had merely assumed that the ocular mosaic is uniform, or had been ignorant of its being otherwise. Then the direct visual acuity curve in Fig. 2 could have been assumed as the integral distribution curve. Its first differential would have been considered as the frequency distribution of the thresholds which have to be assumed to account for the data. It is only because we wished to correlate structure and function that we performed the experiments in which various portions of the eye were painted out. The more extended analysis here presented then became necessary.

4. There remain some minor aspects of the data to be considered. Studies on other arthropods have shown that the pigment in and between the ommatidia migrates, and that this migration is controlled by the illumination. The position of the pigment between the ommatidia may affect the degree to which these are isolated from one another, and may thus influence visual acuity. It is difficult to estimate the magnitude of this factor because we know of no data for the bee's eye. Bennitt's (1924) work, however, would indicate that it may be comparatively negligible because, in the arthropods which he studied, the migration begins at quite low illuminations, and is complete over a very restricted increase in intensity. If the bee's eye behaves in a similar manner, pigment migration would hardly enter to distort the relationship shown in Fig. 7.

Another matter concerns the shape of the bee's eye. Our measurements relate only to a uni-dimensional resolution on the eye. The frequency curves in Figs. 7 and 8 therefore concern the distribution of functional elements in one dimension only. It might then seem that, though useful as description, such curves cannot represent any-

thing basic in the constitution of the ocular mosaic. Since the elements in the eye end in a surface, it might appear that a frequency distribution per unit area would be more fundamental than a distribution per unit distance. The simplest method of converting a linear distribution into a surface distribution would be, in the case of a uniform mosaic, to consider the square of the linear distribution.

There are however several reasons which render it probable that a linear frequency distribution is nearer reality than a surface one. The eye is about four times as long as it is wide. Furthermore the angular separation between adjacent ommatidia is more than three times as great in the horizontal meridian as in the vertical meridian. Both these facts would tend to make the bee's eye an organ which functions essentially as a linear receptor.

This is indeed true experimentally. Baumgärtner found that bees in the field are very astigmatic, and resolve their environment vertically with much greater accuracy than horizontally. Our own experiments showed this independently. Our first tests were made with a long creeping compartment in which the bee crawled from one end to the other, and we tried to make it slow up or turn around by moving a set of stripes under it either in the direction in which the bee was creeping or in the opposite direction. We got no reliable results this way. It was only when the apparatus was changed so that the bee crawled in a direction perpendicular to the movement that we secured significant results. The bee's eye thus seems to be functionally and structurally an organ concerned with the resolution of the environment mainly in one dimension. The data, and the statistical curves in Figs. 7 and 8, therefore very likely represent the real rather than an apparent distribution of elements in the ocular mosaic.

SUMMARY.

1. Bees respond by a characteristic reflex to a movement in their visual field. By confining the field to a series of parallel dark and luminous bars it is possible to determine the size of bar to which the bees respond under different conditions and in this way to measure the resolving power or visual acuity of the eye. The maximum visual acuity of the bee is lower than the lowest human visual acuity. Un-

der similar, maximal conditions the fineness of resolution of the human eye is about 100 times that of the bee.

2. The eye of the bee is a mosaic composed of hexagonal pyramids of variable apical angle. The size of this angle determines the angular separation between adjacent ommatidia and therefore sets the structural limits to the resolving power of the eye. It is found that the visual angle corresponding to the maximum visual acuity as found experimentally is identical with the structural angular separation of adjacent ommatidia in the region of maximum density of ommatidia population. When this region of maximum ommatidia population is rendered non-functional by being covered with an opaque paint, the maximum visual acuity then corresponds to the angular separation of those remaining ommatidia which now constitute the maximum density of population.

3. The angular separation of adjacent ommatidia is much smaller in the vertical (dorso-ventral) axis than in the horizontal (anterio-posterior) axis. The experimentally found visual acuity varies correspondingly. From this and other experiments as well as from the shape of the eye itself, it is shown that the bee's eye is essentially an instrument for uni-directional visual resolution, functional along the dorso-ventral axis. The resolution of the visual pattern is therefore determined by the vertical angular separation of those ocular elements situated in the region of maximum density of ommatidia population.

4. The visual acuity of the bee varies with the illumination in much the same way that it does for the human eye. It is low at low illuminations; as the intensity of illumination increases it increases at first slowly and then rapidly; and finally at high intensities it becomes constant. The resolving power of a structure like the bee's eye depends on the distance which separates the discrete receiving elements. The data then mean that at low illuminations the distance between receiving elements is large and that this distance decreases as the illumination increases. Since such a moving system cannot be true anatomically it must be interpreted functionally. It is therefore proposed that the threshold of the various ommatidia are not the same but that they vary as any other characteristic of a population. The visual acuity will then depend on the distance apart of those elements

whose thresholds are such that they are functional at the particular illumination under investigation. Taking due consideration of the angular separation of ommatidia it is possible to derive a distribution curve for the thresholds of the ommatidia which resembles the usual probability curves, and which describes the data with complete fidelity.

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THE CONCENTRATION EFFECT IN NITELLA.*

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Different concentrations of the same salt brought in contact with living tissue commonly show a P.D. which is known as the concentration effect. Since it can exist only when the surface is permeable to ions¹ it is obviously important for the study of permeability provided we can distinguish between effects due to the living protoplasm and those due to cell walls or other non-living structures. This viewpoint occasioned the present investigation. It early became apparent that in order to interpret the results a picture of conditions in the cell must be worked out which has necessitated additional experimentation.

Quantitative studies of concentration effects were first made by MacDonald,² experimenting on nerves but without distinguishing carefully between living and dead cells. The theoretical implications of such measurements were first pointed out by Loeb and Beutner.³ They investigated plant organs with a cuticularized cell wall which may have been largely responsible for the observed effects;⁴ their experiments on muscle,⁵ like those of Matsuo⁶ on frog liver, gave very little con-

* The authors desire to express their gratitude to the Carnegie Institution of Washington, D. C., which generously provided for the beginning of these investigations (1922-25).

¹ Osterhout, W. J. V., *J. Gen. Physiol.*, 1927-28, xi, 83.

² MacDonald, J. S., *Proc. Roy. Soc. London*, 1900, lxxvii, 310; *Thompson Yates Laboratory, Liverpool*, 1902, iv, pt. 2.

³ Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1; xlv, 303; 1913, li, 300. Cf. Beutner, R., *Die Entstehung elektrischer Ströme in lebenden Geweben*, Stuttgart, 1920.

⁴ Beutner³ states that the same results were obtained after the tissue was killed by chloroform. See also Fujita, A., *Biochem. Z.*, 1925, clviii, 11.

⁵ The results of Loeb and Beutner on the finger nail and the skin are probably due in large part to non-living elements.

⁶ Matsuo, T., *Arch. ges. Physiol.*, 1923, cc, 132.

centration effect. Recent experiments by Jost⁷ have dealt with the concentration effect in *Nitella* without separating the effect due to the protoplasm from that of the cell wall: but in the experiments of Brooks⁸ the effect of the cell wall seems to have been avoided.

Our experiments on *Nitella* have been carried on at various times from 1922 onward. The range of concentrations studied was as a rule from 0.001 M to 0.1 M. Above this plasmolysis and injury are apt to occur and in very dilute solutions there is danger of contamination. The experiments were arranged as in Fig. 1. The temperature averaged about 23°C. Details of technique are given in previous papers.⁹

In all cases the results were recorded photographically and the measurements were made from these records.

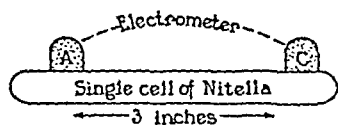


FIG. 1.

FIG. 1. Diagram to show the arrangement of the experiments. Flowing junctions or wads of cotton soaked in solutions are applied at A and C or the ends are allowed to dip into cups containing solutions. Cotton soaked in distilled water is in some cases applied for a short stretch between A and C but a space is left on each side of the cotton to prevent short-circuiting.

It was first necessary to ascertain whether the observed concentration effect is entirely due to the protoplasm or whether the cell wall also plays a part.

In order to study the cell wall dead cells were emptied of sap by cutting off one end and squeezing out their contents. They were then allowed to fill with tap water, and again squeezed out and allowed to fill with tap water. This was repeated several times. Their contents were then squeezed out and they were then filled with air and used at once. In this way it is believed that we may approximate the condition of the cell wall in the living cell where there is probably not much ionic exchange between the cell wall and the protoplasm.

The ends of the dead cell walls (filled with air) were allowed to dip into solutions (in which calomel electrodes were immersed) in such fashion that the solutions did not creep into the lumen (if this occurs it may cause variations in the magnitude or even in the sign of the P.D.). On account of the high resistance the meas-

⁷ Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch., Abt. B*, 1927, Abhandl. 13, Nov.

⁸ Brooks, S. C., and Gelfan, S., *Protoplasma*, 1928, v, 86. (This article was received after the completion of this paper.)

⁹ Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, (a) 1927-28, xi, 673; (b) 1928-29, xii, 167.

P.D. against 0.01 M KCl

Millivolts

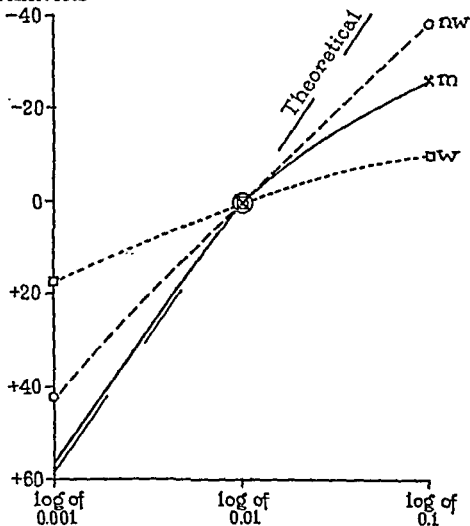


FIG. 2. Curves showing the concentration effect of KCl (each point is the average of 10 to 130 determinations, the probable error of the mean being in general less than 10 per cent of the mean). Curve *m*, concentration effect of the protoplasm alone (i.e., on the *m* basis); Curve *nw*, that of the cell wall and protoplasm combined (i.e., on the *nw* basis); Curve *w*, that of the cell wall alone. The potential difference across the protoplasm when in contact with 0.01 M KCl is taken as zero for purposes of comparison (thus if the curve shows 60 millivolts at 0.001 M KCl it means that this is the potential difference between 0.001 M KCl and 0.01 M KCl). The actual value (called the *AP* value) across the protoplasm in contact with 0.01 M KCl is -2.9 millivolts (average of 60 experiments).

For Curve *m* the values are +56.7 and -25.4; for Curve *nw* +42.2 and -37.9; for Curve *w* +16.8 and -10.

urements on the dead cell wall were made with a Compton electrometer (Cambridge Instrument Co.).

Flowing contacts must be used with caution (especially when the solutions are dilute) on account of "streaming potential" (a good criterion of this is the P.D. observed when identical solutions are placed at the opposite ends, one flowing and the other not, and unless this gave approximately zero the experiment was rejected: in all cases these results were checked by the employment of cups instead of flowing contacts).

Experiments of this kind on cell walls gave the results shown in Curve *w* (Fig. 2). It is therefore evident that the cell wall can give a concentration effect.¹⁰

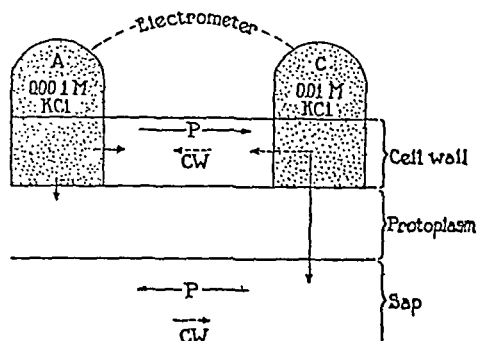


FIG. 3. Hypothetical diagram of conditions in the cell. The arrows show the direction in which the positive current tends to flow and their length the relative magnitude of the P.D. The vertical arrows represent the P.D. due to the protoplasm and the resulting P.D. is labelled *P*; the broken arrows represent the E.M.F. due to the cell wall, and the resulting P.D. labelled *CW*, is opposed to *P*. These relations are more simply represented in Fig. 4.

In order to have some picture of the conditions possibly existing in the cell we may perhaps employ the diagram in Fig. 3 (without by any means assuming that it is correct in all respects). The arrows show the direction in which the positive current tends to flow and their lengths the relative P.D. The vertical arrow at *C* represents the P.D.

¹⁰ In *Nitella* the cell wall is much less cuticularized than the epidermis of the plants used by Loeb and Beutner and it gives a much lower concentration effect.

It may be added that when cells are killed by being placed for 10 minutes in pure chloroform (not in an aqueous solution), removed, and placed in the apparatus they may give a higher concentration effect than the dead cell wall treated as described above.

across the protoplasm: this is opposed by the vertical arrow at A . The arrow at C is longer than that at A since the higher concentration is at C (this would be the case theoretically if the cation has the greater tendency to enter or the greater mobility in the protoplasm) which is in accord with all our measurements.^{9a} The resultant p.d. due to the protoplasm is shown by the arrow labelled P (*i.e.*, the current tends to flow from C through the protoplasm and sap to A and thence through the cell wall to C). (We neglect for the present any short circuit through the protoplasm.)

The relations are represented more simply in Fig. 4 where the resultant resistance of the protoplasm and sap is represented by R_P and that of the cell wall by R_O . The p.d. in the protoplasm is represented by E_{PA} and E_{PC} .

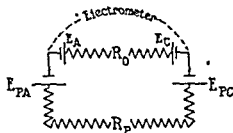


FIG. 4.

FIG. 4. As in Fig. 3 except that the resultant resistance of the protoplasm and sap is represented by R_P (with one horizontal and two vertical components) and that of the cell wall by R_O (having only a horizontal component). The p.d. of the protoplasm is represented by E_{PA} and E_{PC} , that of the cell wall by E_A and E_C .

The relations in the cell wall may be inferred from the fact that when a strip of dead cell wall is used we find a positive current flowing through the electrometer from the dilute to the concentrated solution (which should be the case theoretically if the cation has a greater tendency to enter or a greater mobility than the anion). The p.d. is represented by arrows parallel to the surface (broken arrows) because the applied solution quickly penetrates through the wall (in a few seconds¹¹) and then begins to diffuse along the wall, setting up the p.d. shown by the broken arrows in the figure: the resultant p.d. due to the cell wall is labelled CW and is opposite to that labelled P .

It is therefore evident that the concentration effect of the cell wall

¹¹ This is shown by the fact that substituting 0.01 M NaCl for 0.01 M KCl has practically no effect on the dead cell so that when such a change is made on the living cell we must regard the effect as due entirely to the protoplasm: the chief electrical disturbance due to such a change lasts only a few seconds during which time the solution must have penetrated through the wall to the protoplasm.

tends to increase the values due to the protoplasm. For example, in Fig. 4 (where the broken arrow at C is represented by E_C and that at A by E_A) we may write¹²

$$\text{Obs. conc. effect} = (E_{PC} - E_{PA}) \frac{R_O}{R_O + R_P} + (E_C - E_A) \frac{R_P}{R_O + R_P}$$

Here $E_{PC} - E_{PA}$ corresponds to the arrow labelled P in Fig. 3 and $E_C - E_A$ to that labelled CW . Since R_P is small^{9a} compared to R_O the effect of the cell wall is probably small.

In order to eliminate the action of the cell wall and to ascertain the concentration effect due to the living protoplasm we may apply identical solutions to A and C , and kill C .¹³ If, for example, we lead off at A and C with 0.01 M KCl we eliminate the effect of the cell wall but this gives no P.D. until we kill C (which reduces the E.M.F. of the protoplasm at C approximately to zero¹⁴). We make a photographic record on which we measure the P.D. immediately after the death of C to find the potential difference across the protoplasm at A (we shall call this the AP value).

In order to see what this means let us consider the factors which determine the P.D. We may assume that under normal conditions all the E.M.F. at A is balanced by corresponding E.M.F. at C so that if we lead off from A to C with the same solution the P.D. will be zero. When we change the salt solution at A it seems probable that its penetration is so slow that in our very brief experiments we make no change in the protoplasm except at the outer surface of X .

The electromotive force at the outer surface of X may be called E_{AX} , the corresponding E.M.F. at C being E_{CX} (the E.M.F. in the region lying between A and C , or outside these points, may be neglected for our present purpose). If the E.M.F. at the outer surface of X

¹² It is assumed that the resistance of the electrometer is sufficiently high to preclude any error in measurement and this is the case even when a grid leak is used to shunt the grid and filament (the resistance of the grid leak is 20 megohms).

¹³ As shown in a former paper^{9a} killing C usually produces no immediate effect at A , which can be detected by our present methods.

¹⁴ The killing of C apparently produces no effect on the cell wall. There is, of course, some current flowing in the cell wall but the experiments of Dr. Blinks indicate that this produces no polarization.

in contact with 0.01 M KCl be called $E_{AX\ 0.01\ KCl}$ and all other E.M.F. at A be designated as E_{AZ} the total E.M.F. at A is $E_{AX\ 0.01\ KCl} + E_{AZ}$. What we measure is probably a certain fraction of this which we will call m ,¹⁵ so that the observed P.D. across the protoplasm at A is

$$\text{Observed P.D.} = m(E_{AX\ 0.01\ KCl} + E_{AZ}) = mE_{AX\ 0.01\ KCl} + mE_{AZ}$$

(The E.M.F. in the cell wall at A and C balances out since both spots are in contact with the same solution.¹⁶)

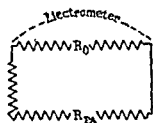


FIG. 5. Hypothetical diagram of electrical resistances in the protoplasm when C is killed. R_0 (with horizontal component only) represents the resistance of the cell wall and R_{PA} (with one vertical and one horizontal component) that of the protoplasm and sap.

FIG. 5.

When we substitute 0.001 M KCl for 0.01 M KCl at A and C we change $E_{AX\ 0.01\ KCl}$ to $E_{AX\ 0.001\ KCl}$ but in brief experiments we may assume that E_{AZ} will not change. If we subtract the observed P.D. across the protoplasm in contact with 0.01 M KCl from that across the protoplasm in contact with 0.001 M KCl we have

$$\text{Observed concentration effect} = mE_{AX\ 0.001\ KCl} - mE_{AX\ 0.01\ KCl}$$

The value of m depends on the resistance of the cell wall (R_0) between A and C and the resultant resistance (R_{PA}) of the protoplasm and sap in the circuit after C is killed. If we represent the resistances as shown in Fig. 5 we are measuring the P.D. across R_0 and the value

¹⁵ This has been discussed in a previous paper^{2a} (where n was used in place of m).

¹⁶ This would not be true if we did not employ a flowing contact at C for sap would come out through the dead protoplasm and change the concentration in the wall, unless the applied solution were sap or 0.05 M KCl or its equivalent. But the flowing contact appears to prevent any disturbance due to the coming out of sap as is shown in a former paper.^{2a} Care must be taken to avoid any disturbances due to "flowing potential."

of m will therefore be $m = R_0 \div (R_0 + R_{PA})$. Measurements¹⁷ made by Dr. Blinks indicate that when C is killed and the solution at A is 0.001 M KCl (or a higher concentration), with the cell wall between A and C imbibed with tap water, the values of R_0 and R_{PA} are such that m is between 0.8 and 1.0.

In order to get some idea of the change in resistance due to killing C we apply 0.01 M NaCl to A and 0.01 M KCl to C (without killing A

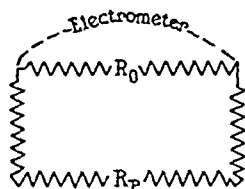


FIG. 6. As in Fig. 5 but with both ends intact (R_P in place of R_{PA}).

FIG. 6.

or C). Since $E_{CX \text{ 0.01 KCl}} = E_{AX \text{ 0.01 KCl}}$ the observed effect (which will be called the chemical effect) may be regarded as

$$\text{Obs. chem. effect} = nE_{AX \text{ 0.01 NaCl}} - nE_{CX \text{ 0.01 KCl}} = nE_{AX \text{ 0.01 NaCl}} - nE_{AX \text{ 0.01 KCl}}$$

where $n = R_0 \div (R_0 + R_P)$ and R_P represents the resultant resistance of the protoplasm and sap in the circuit in the intact cell as indicated in Fig. 6. (The P.D. of the cell wall may be neglected since the cell wall alone gives no chemical effect.)

If we now kill C and ascertain the chemical effect (in the same manner as for the concentration effect, *i.e.*, by measuring the AP value at A of 0.01 M NaCl and then that of 0.01 M KCl) we shall have two measurements, *i.e.*, $m(E_{AX \text{ 0.01 NaCl}} - E_{AX \text{ 0.01 KCl}})$ and $n(E_{AX \text{ 0.01 NaCl}} - E_{AX \text{ 0.01 KCl}})$. From these measurements we find that the value of $m \div n$ is not far from 1 and direct measurements of resistance made by Dr. Blinks indicate that the value of n is not much

¹⁷ We regard R_{PA} and R_0 as resistances in parallel whose resultant resistance is equal to $\frac{(R_{PA})(R_0)}{R_{PA} + R_0}$ which is measured by determining the resistance of the circuit through A and C after C has been killed. We then measure an equal length of cell wall blown up with air (after the protoplasm has been squeezed out) to obtain R_0 . From this we obtain R_{PA} .

below that of m . In other words we compare the values of $m = \frac{R_0}{R_0 + R_{PA}}$ and $n = \frac{R_0}{R_0 + R_P}$ and find that the difference between R_P (the resistance of the protoplasm and sap) and R_{PA} (the same resistance after killing C) is not enough to have any great effect on the values of m and n : this is, of course, due to the very high value of R_0 (according to the measurements of Dr. Blinks the resistance of the cell wall, imbibed with tap water, is considerably over 4 megohms per inch).

These results are checked by applying 0.001 M KCl at A and the same saturated with chloroform at B (Fig. 7): at the same time we

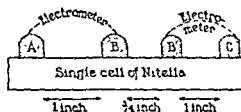


FIG. 7. Diagram to show arrangement of experiments.

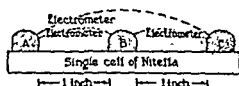


FIG. 8. Diagram to show arrangement of experiments.

apply 0.01 M KCl at B' and the same saturated with chloroform at C (Fig. 7). We thus measure simultaneously the AP value of the two concentrations and by subtracting one from the other obtain the concentration effect. In this case we eliminate both the cell wall effect and errors due to the use of different cells but even so there may be considerable variation. The results agree with those previously described.

Still another method is to arrange the experiments as in Fig. 8 and to kill C with 0.01 M KCl saturated with chloroform. We apply 0.01 M KCl at B and 0.001 M KCl at A . Measuring from A to B gives us the concentration effect of the protoplasm on the n basis together with the cell wall effect: we may call this the nw basis. We

may get the m basis by measuring from B to C and subsequently replacing the 0.01 M KCl at B and C with 0.001 M KCl. We are thus able to measure a single cell on the m and on the mw basis and this may also be done for the chemical effect (n basis) to compare the values of m and n . Here too the results are similar to those already described.

It may be added that when the cell wall is imbibed with 0.001 M KCl and we place 0.01 M KCl at C and 0.001 M KCl at A the results

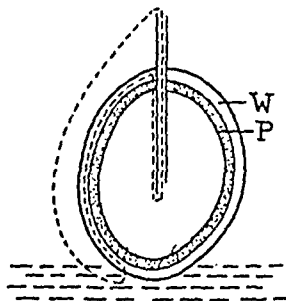


FIG. 9. Diagram to show the electrical leak when a capillary is inserted into *Valonia*.

are practically the same as when the cell wall is imbibed with tap water.

The matter may be approached from another standpoint, *i.e.*, by inserting a capillary. It has been shown in a former paper¹⁸ that when a capillary is inserted into *Valonia* (Fig. 9) we may have a

¹⁸ Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927-28, xi, 193. The use of the capillary in *Valonia* was begun by one of us in the summer of 1923 (for the first reference to this see Osterhout, W. J. V., *J. Gen. Physiol.*, 1924-25, vii, 561). In the summer of 1925 Taylor and Whitaker (Taylor, C. V., and Whitaker, D. M., Carnegie Institution Year Book, 1925-26, No. 25, p. 248) introduced a microelectrode and recently Jost (Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch.*, 1927, Abteilung 13, Nov.) has employed a capillary.

The use of the capillary in *Nitella* was begun by us in 1922 (cf. Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 391). Previous to this Miss Nichols (Nichols, S. P., *Bull. Torrey Bot. Club*, 1925, lii, 351) introduced needles to observe the effect upon structure and upon streaming. Recently Taylor and Whitaker (Taylor, C. V., and Whitaker, D. M., *Protoplasma*, 1927, iii, 1) and Brooks and Gelfan⁸ have employed a microelectrode.

leak at the point of insertion but if we wait for a seal to form this leak disappears and we then may get the true value of the P.D. across the protoplasm. In the case of *Nitella* it is not necessary to wait for a seal to form since the resistance of the cell wall imbibed with tap water is so high that the leak is not important. Hence we may approximate the true values by measuring as soon as the capillary is inserted.

For this purpose the cell is placed on the paraffin block on the stage of a microscope and the capillary (filled with artificial sap) is held firmly in place near the end of the cell. We then seize with forceps a strip of dead cell wall adhering to the

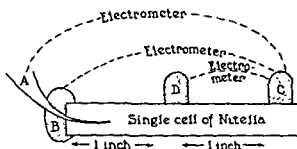


FIG. 10. Diagram to show the arrangement of the experiment when a capillary (A) is inserted in the cell.

end of the living cell (this strip is a part of the neighboring cell whose end has been cut away) and pull the living cell along until it impales itself on the capillary. One can then observe with the microscope whether the capillary is projecting far enough into the vacuole to indicate that its point is not covered by a layer of protoplasm.

An experiment was arranged as shown in Fig. 10. By means of a rotary switch¹⁹ we recorded the circuit from the interior of the capillary A to C (Fig. 11, Curve A, long dash): from the outside of the capillary where it pierced the cell wall, B, to C (Curve B, medium dash) and from D to C (Curve D, short dash). If such an experiment were performed on *Valonia* (Fig. 9) there would be a good deal of difference between A and B provided we had allowed the cell to form an electrical seal but in the case of *Nitella* no such seal is formed as the cell is used immediately after the insertion of the capillary and in consequence there is little difference between A and B.

¹⁹ This was described in a previous paper (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, xi, 391).

The values observed at *A* and *B* (Fig. 11) are a measure of the p.d. across the protoplasm at *C* (*AP* value) as would be the case if we killed with chloroform at *B*. (This *AP* value is negative but appears positive on the record because curves *A* and *B* record the inside of *C* instead of the outside.) The fact that *A* and *B* differ somewhat might be taken to indicate that the point of the capillary has gone through the protoplasm into the vacuole. If it had merely indented the protoplasm, making a deep pocket, without actually piercing it we might expect *A* and *B* to be identical.

The short dash, Curve *D*, records the p.d. of *D* against *C* (which is about 1 millivolt negative); the fact that there is so little differ-

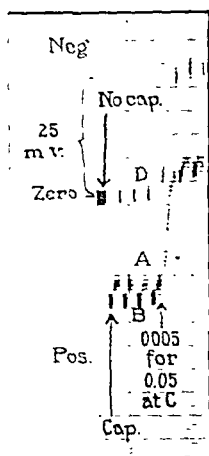


FIG. 11. Photographic record showing potential differences. The experiment is arranged as shown in Fig. 10 with 0.05 *M* KCl at *B*, *D*, and *C*. Before the capillary is inserted there is only one curve (marked "No cap."). As soon as the capillary is inserted the record shows three curves: Curve *A* records the potential difference between the capillary and *C*; Curve *B* that between *B* and *C*; and Curve *D* that between *D* and *C*. When 0.005 *M* KCl is substituted for 0.05 *M* KCl at *C* the curves rise together. The vertical marks represent 5-second intervals.

FIG. 11.

ence between *D* and *C* indicates that injury has not spread from the point of entrance of the capillary to *D* (for when *D* is dead its apparent p.d. will be the same as that of the capillary).

When 0.005 *M* KCl is substituted for 0.05 *M* KCl at *C* both curves rise simultaneously (since *C* becomes more positive which makes both the capillary and *B* appear more negative) to a degree which indicates that *C* is not injured. The amount of this rise is the observed concentration effect which is measured on the equivalent of the *m* basis from *A* to *C* and on the *nw* basis from *D* to *C*. There is evidently no great difference between the two and this might be taken to indicate that the cell wall effect does not greatly alter the value due to the

protoplasm alone. For when we change from 0.05 to 0.005 M KCl at *C* we presumably have a cell wall effect when we measure from *D* to *C* but not when we measure from *A* to *C* or from *B* to *C*, yet the measurement (*i.e.*, the amount of rise) is practically the same in all cases.

Let us now consider certain other aspects of the experiments on *Nitella*. It might be thought that killing *C* tends to alter the concentration effect at *A* (as would be expected if *A* were injured). Hence experiments were made on intact cells having unlike concentrations at *A* and *C* (*i.e.*, on the *nw* basis). The average measurements of 130 such cells (Fig. 2, Curve *nw*) do not agree closely with those made on the *m* basis (58 cells, Fig. 2, Curve *m*), but this lack of agreement seems to have no theoretical significance. On the basis of Figs. 3 and 4 we might expect the values on the *m* basis to be approximately equal to those on the *nw* basis minus the cell wall effect. While this may be approximately true for the value of 0.01 vs. 0.1 M KCl it is just the reverse for the value of 0.01 vs. 0.001 M KCl. In view of this we are not inclined to attribute much quantitative significance to any of these figures.²⁰

Experiments in which *C* was killed (with 0.01 M KCl plus chloroform) and kept in contact with 0.01 M KCl while the solution in contact with *A* was varied (which may be called the *nw* basis, since it includes the effect of the cell wall) gave results much like those in Curve *m*, indicating that the cell wall effect does not greatly affect the results (*i.e.*, curves on the *m* basis do not differ greatly from those on the *nw* basis).

The curves in Fig. 2 are concave to the axis of the abscissæ. Somewhat the same sort of curve was found by Beutner³ in studying organic substances immiscible with water,²¹ although theoretical considerations (to be discussed a little later) lead us to expect in the ideal case the straight line labelled "theoretical" in Fig. 2.

²⁰ We do not know to what extent the values are cut down by short circuits and eddy currents: for example, the current due to the broken arrow at *C*, Fig. 3, might travel in the cell wall only a short distance toward *A* before passing into the protoplasm.

²¹ This is discussed by Michaelis, L., Hydrogen ion concentration, Baltimore, 1926, 200.

In addition to the results shown in Fig. 2 there are a few anomalous cases which should be mentioned. Under certain circumstances the cell may produce a high P.D. (up to 120 millivolts) even with identical solutions at *A* and *C*. It is not surprising therefore that in certain kinds of material we find a concentration effect of 100 millivolts or more for a tenfold dilution (*e.g.*, 0.001 M *vs.* 0.01 M KCl). Since the theoretical limit for a tenfold dilution is about 60 millivolts it is natural to assume that in these anomalous cases the extra P.D. comes from the deeper layers, *Y* or *W*.

In view of such results as those of Michaelis and Fujita²² on gelatin and of Amberson and Klein²³ on dead frog skin, which show a change of sign of the concentration effect with alteration of pH value, it may be well to state that no such change is found in living cells of *Nitella* if in the range studied (pH 5 to 9.5) the concentration of H⁺ is small in comparison to that of other cations.

In view of the fact that organic substances immiscible with water containing organic acids show a concentration effect with KCl with the dilute solution positive while others containing alkalies show the dilute solution to be negative, it may be well to state that somewhat similar results may be obtained by means of a string soaked in acid or alkali, the result being easily accounted for as diffusion potential.

Let us now consider the interpretation of our results. If we regard the P.D. as due primarily to diffusion potential we shall conclude that the mobility of K⁺ in the protoplasmic surface is greater than that of Cl⁻. But if it is due to phase boundary potentials we may

employ the usual formula, putting $R' = \frac{R}{0.4343 n F}$ and $n = 1$

$$\text{P.D.} = R'T \log \left(\frac{C_{coh}}{C_{cxh}} \right) \left(\frac{C_{cxl}}{C_{col}} \right)$$

where C_{cxh} and C_{cxl} are the concentrations of the cations in *X* at the high and low concentrations respectively and C_{coh} and C_{col} are the corresponding concentrations in the external solution. The sign is that of the dilute solution.

²² Michaelis, L., and Fujita, A., *Biochem. Z.*, 1925, clxii, 245.

²³ Amberson, W. R., and Klein, H., *J. Gen. Physiol.*, 1927-28, xi, 823.

If $C_{cxh} = C_{cxi}$ we have

$$\text{P.D.} = R'T \log \frac{C_{coh}}{C_{col}}$$

In metallic electrodes (and in some cases in glass electrodes²⁴) this equation applies but it is a question to what extent this is true of protoplasm.²⁴

In order to picture the relations involved it may be convenient to employ the treatment of Nernst, as discussed in a former paper,¹ where it is stated that if, for example, we apply equimolar solutions of RbCl and CsCl we may write

$$\text{P.D.} = \frac{R'T}{2} \log \frac{A_{Cs}}{A_{Rb}}$$

where A_{Cs} and A_{Rb} are the "true"²⁵ partition coefficients of Cs and Rb. The sign is that of the solution of Rb as shown by the electrometer. If we use two salts without a common ion, *e.g.*, equimolar solutions of RbCl and CsBr, we may take first the phase boundary potential at the spot in contact with RbCl: this is²⁶

$$\text{P.D.} = \frac{R'T}{2} \log \frac{A_{Cl}}{A_{Rb}}$$

the positive sign meaning that the positive current tends to flow from Y to X in the protoplasm.²⁷ The corresponding value for CsBr would be

$$\text{P.D.} = \frac{R'T}{2} \log \frac{A_{Br}}{A_{Cs}}$$

²⁴ This has been discussed in a former paper.¹

²⁵ The true partition coefficient of Rb is the partition coefficient (in this case the concentration at equilibrium in the protoplasmic layer X divided by the concentration in the external solution) it would have if it could enter unhindered by Cl, or in other words if Rb and Cl had the same "true" partition coefficient.

²⁶ For a discussion of this see¹.

²⁷ This is adopted as a convention.

Subtracting one from the other we have

$$\text{P.D.} = \frac{R'T}{2} \log \left(\frac{A_{\text{Cl}}}{A_{\text{Rb}}} \right) \left(\frac{A_{\text{Cs}}}{A_{\text{Br}}} \right)$$

The sign is that of the solution of RbCl, as shown by the electrometer.

We may calculate the concentration effect in similar fashion. If the "true" partition coefficient of the cation is A_{cl} at the low concentration and A_{ch} at the high concentration (the corresponding symbols for the anion being A_{al} and A_{ah}) we may for purposes of illustration make the following substitutions in the last equation: $A_{cl} = A_{\text{Rb}}$, $A_{ch} = A_{\text{Cs}}$, $A_{al} = A_{\text{Cl}}$, and $A_{ah} = A_{\text{Br}}$. We then have

$$\text{P.D.} = \frac{R'T}{2} \log \left(\frac{A_{al}}{A_{cl}} \right) \left(\frac{A_{ch}}{A_{ah}} \right)$$

The sign is that of the dilute solution as shown by the electrometer. It is evident from this equation that in order to get a concentration effect there must be a difference between the "true" partition coefficients of either the cations or of the anions at the two concentrations.

It may be of interest to see how variation of "true" partition coefficients will affect the sign of the dilute solution. If A_c and A_a , as well as $A_c \div A_a$, increase as the concentration increases the tendency of the cations to pass into X will always be greater at the higher concentration so that the positive current will tend to flow from the lower concentration through the electrometer to the higher (dilute solution positive) and at the same time relatively more of the salt will enter at the higher concentration. This would correspond to those cases described by Wosnessensky²⁸ in experiments on amyl alcohol (e.g., the concentration effect of LiCl) where the dilute solution is positive.²⁹

²⁸ Wosnessensky, S., *Z. physik. Chem.*, 1925, cxv, 405; cxvii, 457. Wosnessensky, S., and Astachow, K., *Z. physik. Chem.*, 1925, cxviii, 295; 1927, cxxviii, 362. Wosnessensky, S., Astachow, K., and Tschmutow, K., *Z. physik. Chem.*, 1926, cxxi, 143.

²⁹ In comparing two salts it must be borne in mind that if one is taken up more than the other it does not necessarily mean that in the former case there are more ions in X since the dissociation constants in X may differ. In our discussion we shall assume that the dissociation constants are not very different.

But if $A_c \div A_a$ and A_c increase with concentration while A_a decreases as the concentration increases the dilute solution will still be positive although relatively less salt will be taken up at the higher concentration and this may be true even if both A_c and A_a decrease as the concentration increases.

If on the other hand $A_c \div A_a$ decreases as the concentration increases the dilute solution will be negative even though A_a increases as the concentration increases or though both A_c and A_a increase as the concentration increases (in the latter case the penetration of salt increases as the concentration increases).

We therefore see that on this basis the sign of the dilute solution depends on the behavior of $A_c \div A_a$ and not on the relative amount of salt taken up.

According to recent researches³⁰ and theoretical discussions³¹ little or no P.D. is to be expected with KCl at phase boundaries. On the other hand Michaelis and others³² find fairly high values for the P.D. of KCl with collodion membranes. This might lead to the suspicion that the results found with *Nitella* are largely due to the cell wall but it is evident from what has been said that this is not the case. The concentration effect of the protoplasm is much greater than that of the cell wall and this difference is much more striking in the case of the chemical effect. It should be noted that collodion membranes give the same sort of chemical effect with KCl and NaCl as does protoplasm.

There are difficulties in regarding the P.D. as due to Donnan effect, since the cell cannot very well be in Donnan equilibrium with two different concentrations at the same time. We must assume that the indiffusible ion is positive in order to make the dilute solution positive. The numerical relations may be illustrated as follows: If we have inside an indiffusible anion $R^- = 21$ and outside KCl = 10 we have $(K^+_{\text{outside}})(Cl^-_{\text{outside}}) = 100$: inside the product of K^+ (= 25) and

³⁰ Cf. Holleman, L. W. J., and Werre, J. P., *Rec. trav. chim. Pays-Bas*, 1928, ser. 4, xlvii, 105.

³¹ Cf. Cremer, M., in Bethe, A., *et al.*, *Handbuch der normalen und pathologischen Physiologie*, Berlin 1928, viii, 1034.

³² Cf. Michaelis, L., and others. Various articles in *J. Gen. Physiol.*, 1925-29, ix-xii.

$\text{Cl}^- (= 4)$ is also 100. If we now raise the outside concentration to 100 we have outside (K^+) (Cl^-) = 10,000 and inside the product of $\text{K}^+ (= 111.1)$ and $\text{Cl}^- (= 90.1)$ is also 10,000 (R^- being 21 as before).

In the former case the value of $\frac{C_{co}}{C_{ci}}$ is $\frac{10}{25}$ and in the latter $\frac{100}{111.1}$. We then employ the usual formula

$$\text{P.D.} = R'T \log \frac{C_{co}}{C_{ci}}$$

(where C_{co} is the concentration of cations outside and C_{ci} the concentration inside) which would give for the lower concentration P.D.

$$= R'T \log \frac{25}{10} \text{ and for the higher concentration P.D.} = R'T \log \frac{111.1}{100}$$

so that the concentration effect would be

$$\text{P.D.} = R'T \log \left(\frac{25}{10} \div \frac{111.1}{100} \right) = R'T \log 2.25$$

The dilute solution would be positive but the P.D. would be less than with a metallic electrode where the concentration effect would be $R'T \log 10$ (which at 18°C. would be 58 millivolts). Hence the assumption of a Donnan equilibrium as the explanation of the P.D. may not give values as high as occur in dilute solutions in the case of living cells (whose values approximate those found with metallic electrodes). In order to get values approaching those for metallic electrodes we should have to assume values for R^- more than 10 times as great as those given above.

It is evident that as the more dilute solution of KCl is positive with living protoplasm K^+ tends to enter and the value of $A_e \div A_o$ must increase with the concentration or else the mobility of K in the protoplasm must be greater than that of Cl^- .

There is no direct proof that Cl^- tends to penetrate³³ in ionic form.

Let us now consider the bearing of our results upon electrical conditions in the protoplasm. Adopting the hypotheses set forth in a former paper^{1, 9a} that the protoplasm consists of layers with different

³³ It may reach the vacuole by passing through the non-aqueous layers of the protoplasm in the undissociated condition.

properties we might diagram the electrical conditions as shown in Fig. 12. The X and Y arrows marked A and C under "Net result" at the left have had their directions determined by considerations set forth in a former paper;^{9a} and this is confirmed by the fact that when sap is applied to the outer surface of X (as at the right) the result shows that the X arrow (X_c) is longer than the Y arrow (Y_c).^{9a} This might be regarded as indicating that X gives a greater P.D. with sap than does Y in which case we might make the arrow at the external surface of X longer than that at the inner surface of Y , as is done in the figure: we should in that case be inclined to suppose that X gives a greater P.D. against W than does Y and to make the arrows at the outer and inner surfaces of W of corresponding length (as in the figure). The arrows at b , c , and d will be of the same length at A as at C no matter what the external solutions may be, since in

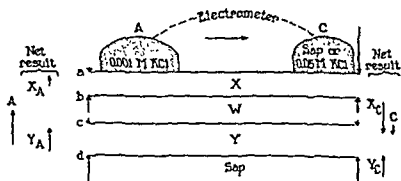


FIG. 12. Hypothetical diagram to show the condition of the protoplasm in contact with different concentrations of KCl. The direction of the arrows indicates how the positive current tends to flow and their length the relative P.D.

brief experiments such as we perform the arrow at a is the only one to change, except in case of injury.

If the electrolyte in W should happen to be mostly KCl the fact that with the protoplasm in contact with 0.001 M KCl the arrow at a is shorter than that at b would mean that the concentration of KCl in W was greater than 0.001 M . But if the electromotive forces at b and c are chiefly due to the diffusion potential, for example of an organic substance produced in the protoplasm, we cannot draw any conclusions regarding the concentration of inorganic salts in W .

If we suppose that X and Y are non-aqueous layers and that W is aqueous we may surmise that each surface is the seat of electromotive

forces as shown in Fig. 12. When we apply 0.001 M KCl at *A* and 0.1 M KCl at *C* it is probable that under normal conditions the E.M.F. at *A* and *C* is equal and opposite except those at the outer surface which are represented in the diagram by arrows of unequal length. If we regard the observed effects as due to diffusion potentials we should say that the cations appear to have a greater mobility in *X* than the anions and consequently we should expect the arrows at the outer surface of *X* to be directed inward, as shown in Fig. 12. The magnitude of the E.M.F. would increase with the concentration, hence the outermost arrow should be longer at *C* than at *A* and the current should flow through the electrometer from *A* to *C* (as shown by the horizontal arrow in Fig. 12). This is in accord with the experimental results which show that the dilute solution is positive not only with KCl but with many other salts. To judge from the literature this is the usual condition³⁴ (but it does not seem to be the case with *Valonia*).

It follows that if we lower the concentration of the solution at *A* we shorten the length of the arrow at *a* and if we could make it equal to that at *b* we should have $a - b = 0$ and we could then learn the value of $c - d$. Whether we can tell when such a state is reached must be decided by future investigation.

The study of the concentration effect may be of value as throwing light on the nature of protoplasm. According to some authors a salt may give no concentration effect with certain substances immiscible with water²⁸ but with others^{35,36} the dilute solution appears positive and with still others negative. If this be the case we may hope by such studies to gain some idea of the nature of the substance constituting the protoplasmic surface.

SUMMARY.

A method distinguishing between the concentration effect due to the cell wall and that due to the protoplasm is described: the importance

³⁴ Cf. Höber, R., *Physikalische Chemie der Zelle und der Gewebe*, Leipsic, 6th edition, 1926.

³⁵ Cf. e.g. Beutner, R., *Die Entstehung elektrischer Ströme in lebenden Geweben*, Stuttgart, 1920.

³⁶ Cf. Cremer, M., in Bethe, A., *et al.*, *Handbuch der normalen und pathologischen Physiologie*, Berlin 1928, viii, 999.

of this lies in the fact that if the protoplasm shows a concentration effect one or both ions of the salt must tend to enter its outer surface.

Studies on the concentration effect of KCl with living protoplasm of *Nitella* show that when P.D. is plotted as ordinates and the logarithm of concentration as abscissæ the graph is not the straight line demanded in the ideal case by theory but has less slope and is somewhat concave to the axis of the abscissæ.

With a variety of salts the dilute solution is positive, which indicates that the cation has a greater mobility in the protoplasm than the anion or that the partition coefficient of the cation (A_c) increases faster than that of the anion (A_a) as the concentration increases. If the result depended on the partition coefficients we should say that when $A_c \div A_a$ increases with concentration the dilute solution is positive. When $A_c \div A_a$ decreases as the concentration increases the dilute solution is negative. In either case the increase in concentration may be accompanied by an increase or by a decrease in the relative amount of salt taken up. Theoretically therefore there need be no relation between the sign of the dilute solution and the relative amount of salt taken up with increasing concentration.

Hypothetical diagrams of the electrical conditions in the cell are given.

If we define the chemical effect as the P.D. observed in leading off at two points with equivalent concentrations of different salts we may say that the chemical effect of the protoplasm is very much greater than that of the cell wall.

CHEMICAL ANTAGONISM OF IONS.

IV. EFFECT OF SALT MIXTURES ON GLYCINE ACTIVITY.

By HENRY S. SIMMS.

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(Accepted for publication, April 15, 1929.)

I.

INTRODUCTION.

The three previous papers of this series¹ demonstrated that Na^+ ions counteract the anomalous effect of Mg^{++} ions on the activity of oxalate di-ions ($\text{C}_2\text{O}_4^{--}$); and also that Cl^- ions counteract the effect of SO_4^{--} ions on oxalate di-ions. Furthermore, NaCl , KCl , or MgCl_2 each affect gelatin activity in the same manner when only one of these salts is present. Each causes a *lowering* of the pH. However, the addition of a small amount of NaCl or KCl to a gelatin solution already containing another of the above salts causes a very marked *rise* in pH (an effect opposite to that produced by one salt alone). This occurs up to 0.10 molar Na^+ or K^+ ion concentration. Further additions cause a *sharp lowering of pH up to 0.15 molar Na^+ or K^+* . Beyond that there is little effect.

The analogy of these observations to physiological antagonisms is obvious. In order to investigate the mechanism it is desirable to see if still simpler substances than gelatin behave in the same way. In the present paper it will be shown that NaCl , KCl , MgCl_2 , and CaCl_2 , singly and in mixtures, affect the activity of glycine (a simple amino acid) in practically the same manner that they affect gelatin activity.

II.

RESULTS.

The results obtained in these experiments are obvious from the accompanying figures. Fig. 1 shows the influence of salts on the pH

¹ Simms, H. S., *J. Gen. Physiol.*, 1928, xii, 241, 259; 1929, xii, 511.

of a 0.0100 molar solution of glycine containing one-half equivalent of NaOH. NaCl alone or KCl alone *lower* the pH in the manner shown. However, if we start with 0.00416 molar KCl and add increasing amounts of NaCl, we get first a *rise* in pH until 0.007 M NaCl is present. Then there is a sharp break and further additions *lower* the pH until 0.035 M NaCl is present. Further additions of NaCl *increase* the pH as shown.

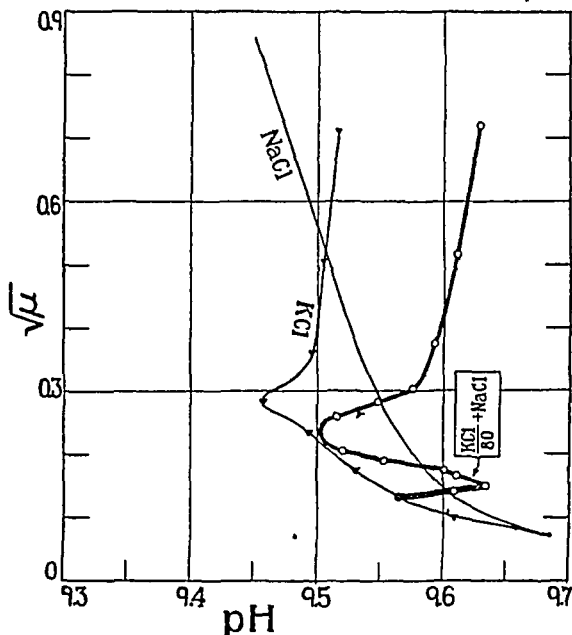


FIG. 1. Antagonistic effects of KCl + NaCl mixtures compared with the effects of KCl or NaCl alone on the pH of glycine solutions (containing 0.5 equivalent of NaOH). Note that both cations are monovalent.

Fig. 2 shows that the same phenomena take place when NaCl is added to glycine solutions containing $MgCl_2$. The breaks come at 0.015 molar and 0.085 molar NaCl concentration *regardless of the amount of $MgCl_2$ present*. This is the same result that was observed with gelatin.

Fig. 3 shows that $CaCl_2$ + NaCl mixtures behave essentially the same as $MgCl_2$ + NaCl mixtures and KCl + NaCl mixtures.

Fig. 4 shows the effect of adding $CaCl_2$ to glycine solution containing $MgCl_2$. The same sort of curve is produced as is found with the other

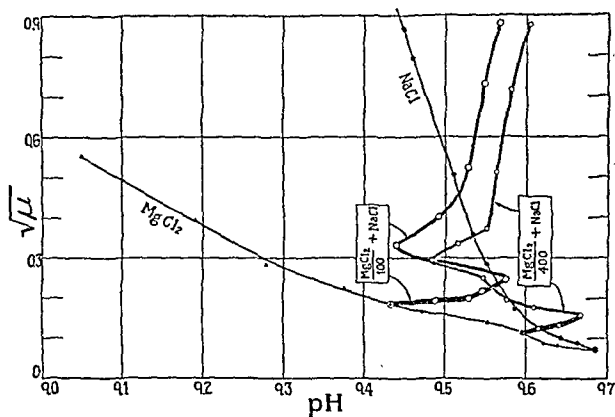


FIG. 2. Effect of $MgCl_2 + NaCl$ mixtures on the pH of glycine solutions, compared with the effect of each salt alone. One divalent and one monovalent cation.

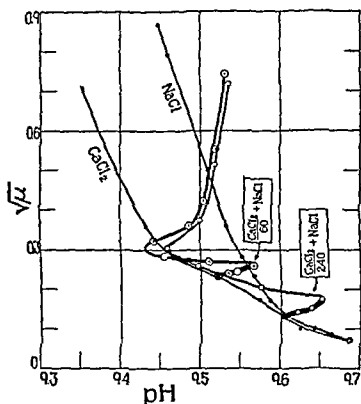


FIG. 3. Effect of $CaCl_2 + NaCl$ mixtures on the pH of glycine solutions, compared with the effect of each salt alone. One divalent and one monovalent cation.

salt mixtures. In Fig. 4 it is noteworthy that after the first break the curve for the mixture of MgCl_2 and CaCl_2 becomes almost identical with that for MgCl_2 alone. After the second break it swings to the right and there is a third break near where it crosses the curve for CaCl_2 alone. There appears to be a tendency toward the same phenomena in Figs. 1, 2, and 3.

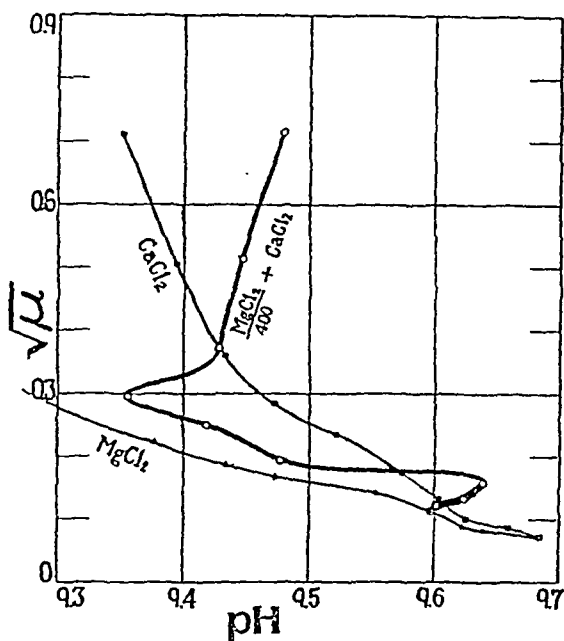


FIG. 4. Effect of $\text{MgCl}_2 + \text{CaCl}_2$ mixtures on the pH of glycine solutions. Both cations are divalent.

In Fig. 5 the curves for the salt mixtures $\text{KCl} + \text{NaCl}$; $\text{MgCl}_2 + \text{NaCl}$; and $\text{CaCl}_2 + \text{NaCl}$ are plotted in another manner. The ordinates are the square root of the Na^+ ion concentration (including the NaOH added to the mother solution). The abscissas are the difference between the observed pH and that obtained with the same ionic strength with NaCl alone. In other words the curve for NaCl is taken as a standard, the deviations from which are plotted in Fig. 5.

It will be observed that the first break of each curve (except the $\text{KCl} + \text{NaCl}$ curve) comes at 0.015 M NaCl concentration *regardless of the amount of the other salt*.

The second break for the MgCl_2 curves is at 0.085 M NaCl and is a little lower for the CaCl_2 curves.

The $\text{KCl} + \text{NaCl}$ curve has its breaks at about one-half the NaCl concentrations at which they occur with $\text{MgCl}_2 + \text{NaCl}$, or $\text{CaCl}_2 + \text{NaCl}$.

The $\text{MgCl}_2 + \text{CaCl}_2$ curve (of Fig. 4) was not plotted in the manner of Fig. 5. However, the first break is at 0.005 M Ca^{++} and the second

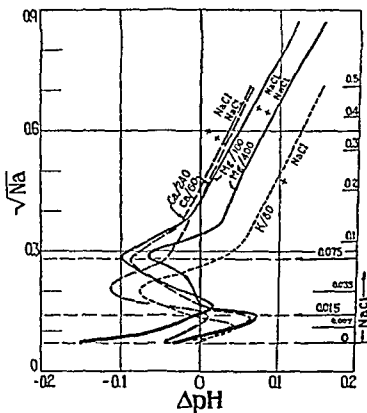


FIG. 5. Effect of NaCl in the presence of another salt, relative to the effect of NaCl alone. The breaks are independent of the amounts of the other salts.

break is at 0.025 M Ca^{++} . It was stated above that the breaks with $\text{MgCl}_2 + \text{NaCl}$ were at 0.015 M and 0.085 M NaCl, respectively. These two sets of values do not agree.

If, however, we convert the ion concentrations into "ionic strength" values we have the breaks with $\text{MgCl}_2 + \text{NaCl}$ coming at $0.015 \mu\text{NaCl}$ and $0.085 \mu\text{NaCl}$, while with $\text{MgCl}_2 + \text{CaCl}_2$ they come at $0.015 \mu\text{CaCl}_2$ and $0.075 \mu\text{CaCl}_2$. Apparently the position of the breaks is a function of the ionic strength of the added salt rather than of its concentration.

These results with glycine cannot be explained on the basis of colloidal phenomena. They tend to support the mechanism suggested

in the previous paper of the series, namely, that cations in mixtures selectively inactivate the two ionic species² to degrees not proportional to the inactivation by one cation alone.

III.

EXPERIMENTAL.

0.02 molar glycine plus 0.500 equivalent of NaOH was made up in 100 cc. amounts. These were used as mother solutions. For each observation 5.00 cc. of mother solution was placed in a 10 cc. volumetric flask, together with desired amounts of salt solutions, and made up to 10 cc. (*i.e.*, 0.0100 molar with respect to glycine). The salts were added from stock solutions of three different ionic strengths (0.025μ , 0.25μ , and 2.50μ). The pH of each solution was measured at 25°C. in a water-jacketed hydrogen electrode of the bubbling type.

Solutions free from salt had a pH of 9.685.

The data are given in Tables I to VIII.

² The two ionic species in the case of glycine in this pH range are, first the *neutral* (or zwitterion) form which predominates at neutral pH values; and second the *anion* form which predominates in alkaline solutions. The lowering of the pH on the addition of a single salt presumably "inactivates" the glycine anion more than the zwitterion. We do not measure the actual inactivation, but rather the ratio of inactivation of the two ionic species. It is reasonable to suppose that this ratio may be disturbed in a mixture of salts.

(Summary on page 792.)

TABLE I.
Effect of NaCl on Na Glycinate.
 $(\mu = C_{\text{NaCl}} + 0.005.)$

C_{NaCl}	pH	C_{NaCl}	pH	C_{NaCl}	pH
0	9.685	0.050	9.567	0.625	9.460
0.0025	9.663	0.075	9.551	0.750	9.448
0.0050	9.643	0.125	9.533	1.000	9.416
0.0250	9.587	0.250	9.510		

TABLE II.
Effect of KCl on Na Glycinate.
 $(\mu = C_{\text{KCl}} + 0.005.)$

C_{KCl}	pH	C_{KCl}	pH	C_{KCl}	pH
0	9.685	0.025	9.526	0.250	9.504
0.0025	9.558	0.050	9.494	0.500	9.516
0.0050	9.609	0.075	9.457		
0.0125	9.565	0.125	9.497		

TABLE III.
Effect of MgCl_2 on Na Glycinate.
 $(\mu = 3 C_{\text{MgCl}_2} + 0.005.)$

C_{MgCl_2}	pH	C_{MgCl_2}	pH	C_{MgCl_2}	pH
0	9.685	0.0050	9.553	0.0250	9.279
0.00050	9.638	0.0075	9.472	0.0500	9.191
0.00100	9.621	0.0100	9.433	0.100	9.049
0.00250	9.596	0.0150	9.376		

TABLE IV.
Effect of CaCl_2 on Na Glycinate.
 $(\mu = 3 C_{\text{CaCl}_2} + 0.005.)$

C_{CaCl_2}	pH	C_{CaCl_2}	pH	C_{CaCl_2}	pH
0	9.685	0.0083	9.574	0.083	9.394
0.00083	9.658	0.0167	9.521	0.167	9.352
0.00167	9.624	0.0250	9.472		
0.00417	9.604	0.0417	9.433		

TABLE V.

Effect of NaCl on a Glycine Solution Containing 0.0125 M KCl.

(pH without salt = 9.685.)

 $(\mu = C_{\text{NaCl}} + 0.0175.)$

C_{NaCl}	pH	ΔpH	C_{NaCl}	pH	ΔpH
0	9.565	-0.045	0.050	9.516	-0.042
0.0025	9.609	+0.005	0.075	9.577	+0.031
0.0050	9.634	+0.034	0.125	9.594	0.064
0.0125	9.601	+0.014	0.250	9.611	0.104
0.0250	9.521	-0.056	0.500	9.628	0.155

TABLE VI.

Effect of NaCl on Glycine Solutions Containing MgCl_2 .

(pH without salt = 9.685.)

C_{NaCl}	pH	ΔpH	C_{NaCl}	pH	ΔpH
A. 0.00250 M MgCl_2 plus various amounts of NaCl ($\mu = C_{\text{NaCl}} + 0.0125$).					
0	9.596	-0.034	0.075	9.484	-0.064
0.0025	9.616	-0.003	0.100	9.516	+0.021
0.0050	9.641	+0.029	0.125	9.550	+0.020
0.0125	9.667	+0.070	0.250	9.563	+0.056
0.0187	9.610	+0.023	0.500	9.582	+0.110
0.0250	9.577	-0.003	0.750	9.604	+0.157
0.0500	9.546	-0.016			
B. 0.0100 M MgCl_2 plus various amounts of NaCl ($\mu = C_{\text{NaCl}} + 0.035$).					
0	9.433	-0.151	0.075	9.440	-0.099
0.0025	9.489	-0.093	0.125	9.491	-0.034
0.0050	9.530	-0.048	0.250	9.528	+0.022
0.0125	9.547	-0.025	0.500	9.548	+0.078
0.0250	9.575	+0.012	0.750	9.567	+0.122
0.0500	9.486	-0.062			

TABLE VII.
Effect of NaCl on Glycine Solutions Containing CaCl_2 .
 (pH without salt = 9.685.)

C_{NaCl}	pH	ΔpH	C_{NaCl}	pH	ΔpH
A. 0.00416 M CaCl_2 plus various amounts of NaCl ($\mu = C_{\text{NaCl}} + 0.0175$).					
0	9.604	-0.006	0.050	9.497	-0.061
0.0025	9.621	+0.017	0.075	9.458	-0.088
0.0050	9.638	+0.038	0.125	9.500	-0.030
0.0125	9.651	+0.064	0.250	9.518	+0.011
0.0250	9.575	-0.002	0.500	9.536	+0.063
B. 0.0167 M CaCl_2 plus various amounts of NaCl ($\mu = C_{\text{NaCl}} + 0.050$).					
0	9.521	-0.046	0.050	9.443	-0.097
0.0025	9.535	-0.030	0.075	9.486	-0.047
0.0050	9.546	-0.017	0.125	9.504	-0.018
0.0125	9.567	+0.010	0.250	9.520	+0.019
0.0187	9.511	-0.021	0.500	9.532	+0.064
0.0250	9.455	-0.096			

TABLE VIII.
Effect of CaCl_2 on Na Glycinate Solutions Containing 0.0025 M MgCl_2 .
 (pH without salt = 9.685.)
 ($\mu = 3 C_{\text{CaCl}_2} + 0.0125$.)

C_{CaCl_2}	pH	C_{CaCl_2}	pH	C_{CaCl_2}	pH
0	9.596	0.0083	9.476	0.083	9.447
0.00083	9.602	0.0167	9.418	0.167	9.479
0.00167	9.624	0.025	9.355		
0.00417	9.639	0.0417	9.428		

SUMMARY.

The pH of a 0.01 molar solution of glycine, half neutralized with NaOH, is 9.685. Addition of only one of the salts NaCl, KCl, MgCl_2 , or CaCl_2 will *lower* the pH of the solution (at least up to 1μ).

If a given amount of KCl is added to a glycine solution, the subsequent addition of increasing amounts of NaCl will first *raise* the pH (up to 0.007 M NaCl). Further addition of NaCl (up to 0.035 M NaCl) will *lower* the pH, and further additions slightly raise the pH.

The same type of curve is obtained by adding NaCl to glycine solution containing MgCl_2 or CaCl_2 except that the first and second breaks occur at 0.015 M and 0.085 M NaCl, respectively.

Addition of CaCl_2 to a glycine solution containing MgCl_2 gives the same phenomena with breaks at 0.005 M and 0.025 M CaCl_2 ; or at *ionic strengths* of $0.015\mu\text{CaCl}_2$ and $0.075\mu\text{CaCl}_2$. This indicates that the effect is a function of the ionic strength of the added salt.

These effects are sharp and unmistakable. They are almost identical with the effects produced by the same salt mixtures on the pH of gelatin solutions. They are very suggestive of physiological antagonisms, and at the same time cannot be attributed to colloidal phenomena.

THE EFFECT OF SUDDEN CHANGES OF TEMPERATURE ON PROTOPLASMIC STREAMING.

By S. F. COOK.

(From the Division of Physiology, University of California, Berkeley.)

(Accepted for publication, March 25, 1929.)

I.

In the numerous studies which have been made concerning the effect of temperature on various cellular activities many interesting irregularities in the conduct of cells and tissues have been brought to light. An example of such phenomena is the sudden cessation of protoplasmic streaming in *Nilrella* which occurs when the temperature is suddenly lowered. This response to temperature change has been discussed recently by Lambers (1, 2), who finds that in case of a sudden transition from hot to cold there is a "shock" which causes the streaming to stop instantly. There is then a recovery but the rate of movement normal for a given temperature is not attained for 10 to 20 minutes.

A similar inhibition is known to take place if the *Nilrella* cell is stimulated mechanically, and Fitting (3) has shown that a sudden increase of light intensity brings about the same effect. There are therefore at least three distinct types of stimuli which cause an instantaneous inhibition of streaming in *Nilrella*. A brief investigation of the inhibition due to change in temperature has been made and the results are here reported.

II.

The material used was *Nilrella clavata* which had been secured from a pond in Golden Gate Park, San Francisco, and had been kept in tanks. Single cells were cut out, placed in petri dishes and examined under the low power of the microscope. The rate of streaming was measured by means of a micrometer eye-piece and a stop watch. Individual determinations were made of the time necessary for a particle to travel between two selected points in th

Since the same objective and eye-piece were always used, the measurements on a large number of cells are comparable. The light source was a 40 watt Mazda bulb placed at a fixed distance from the microscope. This insured a constant illumination and a constant light intensity through the condenser. For lower temperatures the cells were immersed in mixtures of ice and water, the proportions of which were so regulated that by frequent stirring a constant temperature might be obtained. The higher temperatures were maintained by keeping the room at approximately the desired point and then using mixtures of warm and cool water. The error of this method was no greater than 0.1° , and since no intervals of less than 5° were investigated (usually the intervals were at least 10°) this error is without significance. In fact the individual variability of the cells is much greater than any error of the method.

The results may be expressed as microns per second. But since most of the data are of a comparative nature there is no need of introducing absolute units. All rates may be expressed as per cent of the normal rate, the latter being taken arbitrarily as the rate at any given temperature.¹

It may be noted in passing that the sensitivity of *Nitella* to mechanical disturbance, mentioned above, is very great. A small jar or blow, such as striking against any hard object, or a bending of the cell, will stop the streaming completely. Special care must therefore be exercised in handling the cells, particularly in transferring them from one dish to another. In these experiments the following precaution was always taken. Before a cell was immersed in water at a new temperature, it was transferred to a second container with water at the previous temperature. If this operation had no effect on the streaming, the cell was then placed at the new temperature. Furthermore a special series of control experiments showed that with due care cells could be moved back and forth from one petri dish to another indefinitely without affecting the rate of streaming.

¹ The extraordinary constancy of the rate of streaming in *Nitella*, at constant temperature, is worthy of note. Measurement of 73 cells showed an average normal rate at 20° of 73.1μ per second. The maximum deviation was 21.3 per cent and the average deviation was 5 per cent, or 3.65μ per second.

III.

If *Nitella* cells are carried from 20°C. to 35°C., there is no cessation of the streaming. There is of course a change of rate, from that characteristic of 20° to that of 35°. This change is very nearly instantaneous. Usually 20 to 30 seconds elapse before the first record can be made, representing time unavoidably lost in manipulating the cell, getting it under the microscope, and focussing. During this half minute or less the new rate has become established. Probably the actual time consumed in bringing the streaming to the new speed is a matter of a few seconds. A similar situation obtains if a cell is allowed to remain at 1° for 30 minutes or more and is then brought to 20°.

The result may be very different when the temperature is lowered abruptly. If the drop is from 35° to 20° the flow continues without interruption but at a slower rate, *viz.*, that rate characteristic of 20°. The effect of such a fall in temperature is similar to that of a rise, since it causes an immediate change in rate; immediate in the sense that the rate becomes constant within 20 seconds. In fact a cell may be alternated between 35° and 20° indefinitely with no change except in the rate of streaming. Suppose, however, that we place the cell in water at 10° instead of 20°. In this case the streaming stops completely and does not begin again for at least 2 minutes. This instantaneous and complete cessation is a very different phenomenon from the mere change of speed observed between 35° and 20°. The difference is also exemplified in the following experiment. If a cell is taken from water at 35°, rapidly dipped into water at 10° and replaced at 35° the streaming likewise is seen to have completely stopped. The exposure to the temperature of 10° may not last longer than half a second. Yet the effect is observed. The stimulus, then, is clearly the sudden transition from the high to the low temperature and not a prolonged exposure at the low temperature.

Another series of experiments extends the above results and demonstrates that the fall in temperature must exceed a certain definite number of degrees in order to produce the effect. In this case the initial temperature was 20°. The cells were dipped for approximately half a second in water at several lower temperatures and were returned to 20° for observation. The fall from 20° to 15° does not stop the

streaming, nor does that from 20° to 10° . But passing from 20° to 1° the streaming ceased in all cells observed. The transition, or critical point, for an initial temperature of 20° then lies between 1° and 10° . In an attempt to locate this critical point more exactly, two facts became evident.

1. There is a decided variability among the cells with respect to the temperature at which they stop streaming. This is shown in Table I and Fig. 1. With an initial temperature of 20° a number of cells were exposed for less than a second to temperatures varying from 3° to 7° . It was found that in all cells the streaming stopped at 3° , but at the other temperatures (4° , 5° , 7°) only part of the cells were so affected. Plotting per cent not affected against temperature we obtain Fig. 1. From these data it may be concluded that for a large number of *Nitella* cells there is a general range of temperature which causes a cessation of streaming.

TABLE I.

Effect of Bringing Cells from 20° to Various Temperatures for 0.5 Second.

Temp.	No. cells	No. cells unaffected	Per cent of cells unaffected
10°	20	20	100
7°	44	36	82
5°	59	40	77
4°	40	14	35
3°	20	0	0

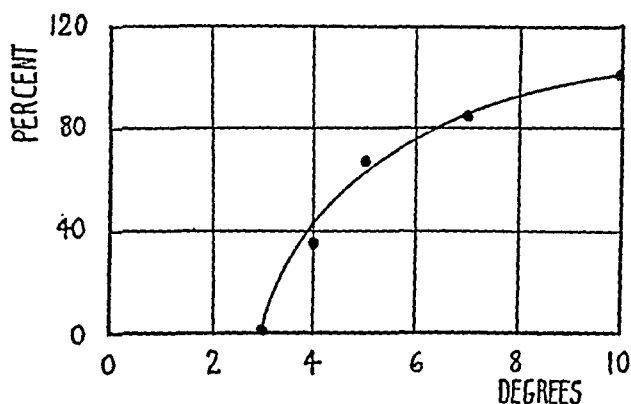


FIG. 1. This figure shows the relative number of cells in which the streaming is inhibited when the initial temperature is 20°C . The ordinates are per cent of the total number in which there is no inhibition. The abscissae indicate the low

2. Nevertheless there exists for every single cell some quite definite critical temperature. In all the cases examined there was no single instance where a partial cessation occurred. The streaming either continued in a normal manner or was inhibited completely.

The data presented thus far permit one more conclusion to be drawn. The inhibition of streaming does not occur at one particular point on the temperature scale regardless of the original temperature, but is rather a function of the temperature gradient. It may be pointed out again that, regardless of the variability of individual cells, a drop from 35° to 10° causes 100 per cent inhibition whereas a drop from 20° to 10° causes no inhibition whatever. Were there any physical or chemical state inherent in the condition of the cell adjusted to 10° which caused an inhibition of streaming, then that state would have to exist regardless of the previous temperature. But since the effect of exposure to 10° is 100 per cent different depending on the previous temperature we are forced to the conclusion that it is the sudden fall of temperature through at least a minimum number of degrees which gives rise to the observed effects. The temperature range with which it is possible to work is too limited to permit an exact quantitative determination of this minimum distance for all initial temperatures, but it may be stated that probably a fall of 15° to 20° is sufficient to stop streaming.

Certain theoretical considerations are pertinent at this juncture. The immediate increase in the rate of streaming with sudden rise in temperature, and, within the prescribed limits, the corresponding decrease with fall of temperature, point to the acceleration and retardation of a chemical reaction system. Since the streaming in these cells is considered to be governed by their metabolism it is entirely reasonable that these particular effects of temperature should be obtained. It is what might be expected of a chemical system. But the discontinuity introduced by the sudden cessation of streaming below the critical temperature cannot be explained on such a basis. It is very unlikely that a chemical reaction can be brought to a complete stop by a fall of 20° . This view is substantiated by the fact that there is no intergradation between moderate retardation and complete inhibition. We must therefore fall back on the supposition that the inhibition is a phenomenon which has no connection with the metabolism of the cell

and which is connected with the physical properties of the protoplasmic system.

IV.

If cells are transferred from 20° to 1° and allowed to remain at 1° for varying periods before being returned to 20° there is a distinct influence of the exposure time on the recovery of the normal rate. This is illustrated in Figs. 2 to 5. Each figure represents data from several cells. The individual variability causes considerable dispersion of the points, but if enough cells are studied it is found that the dispersion falls within definite limits, forming a band of points instead of a single curve. For practical purposes, however, a curve may be drawn along the midline of the band which will represent an average of all the points.

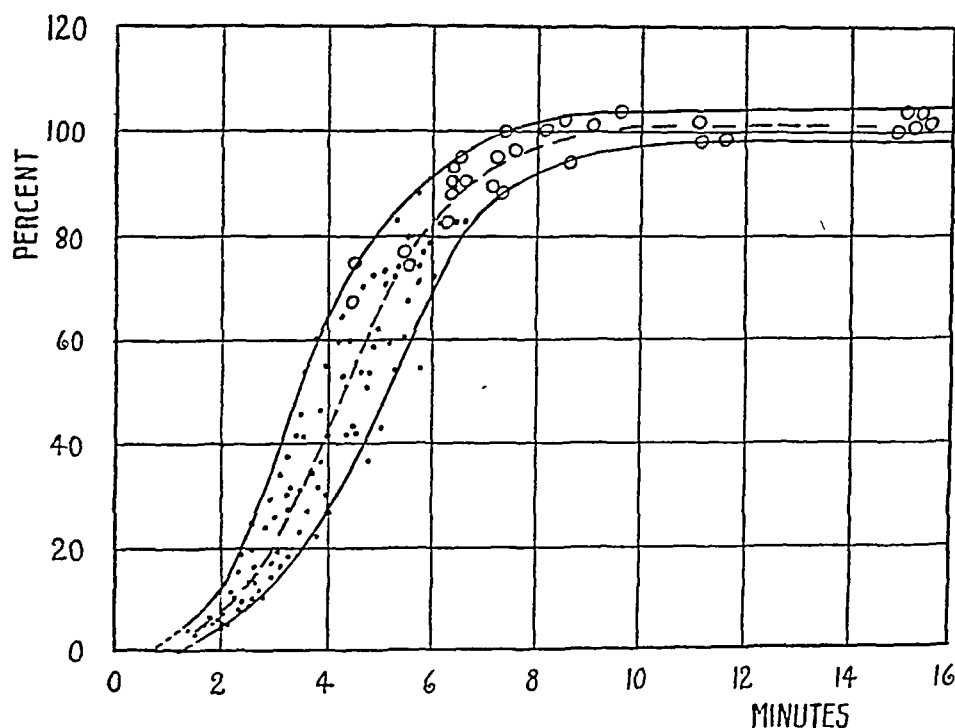


FIG. 2. The course of recovery of streaming in 9 cells after 0.5 second exposure to 1°C . The initial temperature was 20°C . and the cells were replaced at this temperature for measurement. The ordinates denote per cent of the normal rate, the abscissae time in minutes. The dots represent single measurements, the circles represent averages of at least three measurements.

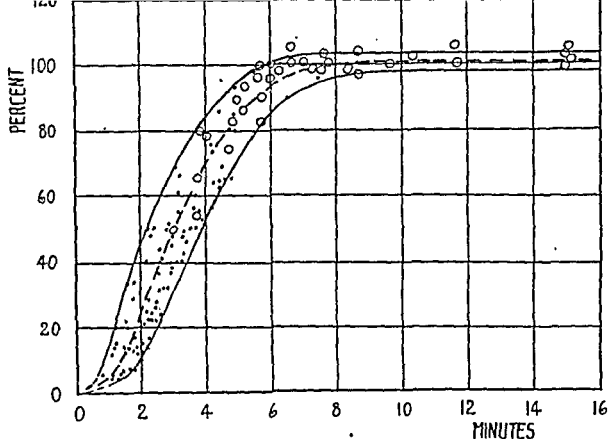


FIG. 3. The course of recovery of 10 cells after 5 minutes exposure to 1°C . Initial temperature 20°C . Ordinates and abscissae as in Fig. 2.

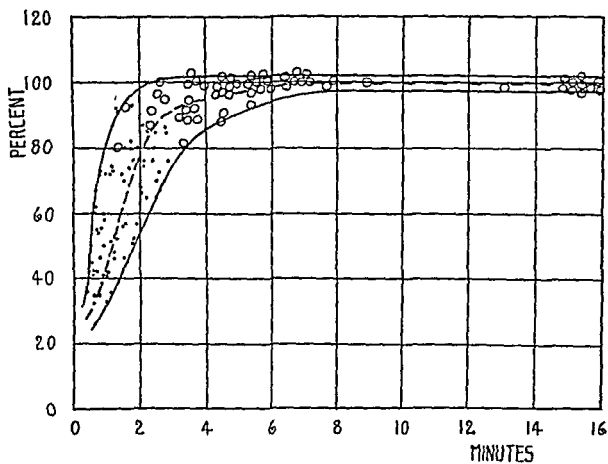


FIG. 4. The course of recovery of 12 cells after 10 minutes exposure to 1°C . Initial temperature 20°C . Coordinates the same as Fig. 2.

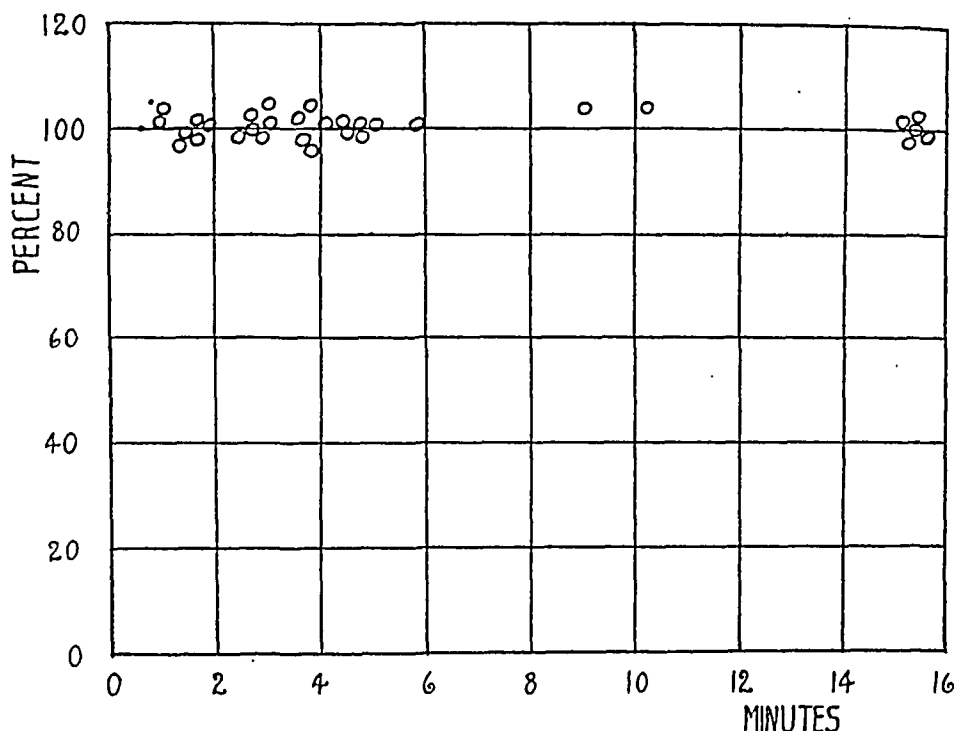


FIG. 5. The course of recovery of 6 cells after 20 minutes exposure to 1°C. Initial temperature 20°C. Coordinates as in Fig. 2.

It is evident from an inspection of these figures that after a short exposure (0.5 second in Fig. 2) to the low temperature there is at first a latent period during which there is no perceptible motion. After 1 to 2 minutes the streaming begins slowly, increases its rate rapidly, and soon returns to the normal. After a 5 minute exposure approximately the same course of recovery is observed, with the exception that the latent period is shorter, but after a 10 minute exposure the return to the normal is very rapid, with no latent period. If the exposure is of 20 minutes duration the resumption of the normal rate is immediate, and resembles the behavior of the cell when it is carried from 20° to 35°. In other words the longer the exposure to the low temperature the more rapid the recovery when the cell is replaced at the higher temperature.

These results may be explained as follows. The same course of

recovery is followed at all temperatures; the rate varies with the temperature. There is at first a latent period prior to actual recovery. This lasts about a minute when the cell is at 20° and probably about 5 minutes at 1° . Evidence for this is the fact that the initial absence of motion at 20° continues much longer when the cell has been exposed to 1° for 0.5 second (Fig. 2) than it does when the exposure period is 5 minutes. In fact, judging by the curve in the latter case (Fig. 3) there can be scarcely any latent period at all. Thus the latent period has practically come to an end when the cell is replaced at 20° . On the other hand the remaining portions of the two curves are nearly identical, and nearly the same time is consumed for the streaming to return to the normal rate.

If the cell is removed from 1° at the end of 10 minutes the actual, rapid, recovery is in progress. This is demonstrated quite clearly by the fact that (Fig. 5) after 20 minutes the resumption of the normal rate for 20° is immediate. Therefore we may say that after the streaming has been brought to a complete standstill by the sudden exposure to a temperature of 1° the recovery starts after a latent period, and follows an S-shaped course until it is complete. If the cell remains at 1° this sequence of events is carried out relatively slowly. If the cell at any time during the process is replaced at 20° the recovery proceeds at a more rapid rate than that prior to the moment of replacement. Hence recovery at 20° is *apparently* quicker the longer the exposure to the low temperature.

v.

The statement was made previously that the inhibition of streaming at low temperatures might have a physical explanation. The data on recovery afford two more lines of evidence concerning this point.

1. Even though, at 1° , recovery is proceeding, a very careful examination for over 30 minutes failed to detect any perceptible motion in the protoplasm. Recovery of the *power to stream* can thus take place while there is no *actual streaming* in progress. Therefore the two factors, the actual streaming, and the capacity for streaming, must be entirely separate and distinct.

2. We may determine roughly the temperature coefficient of the

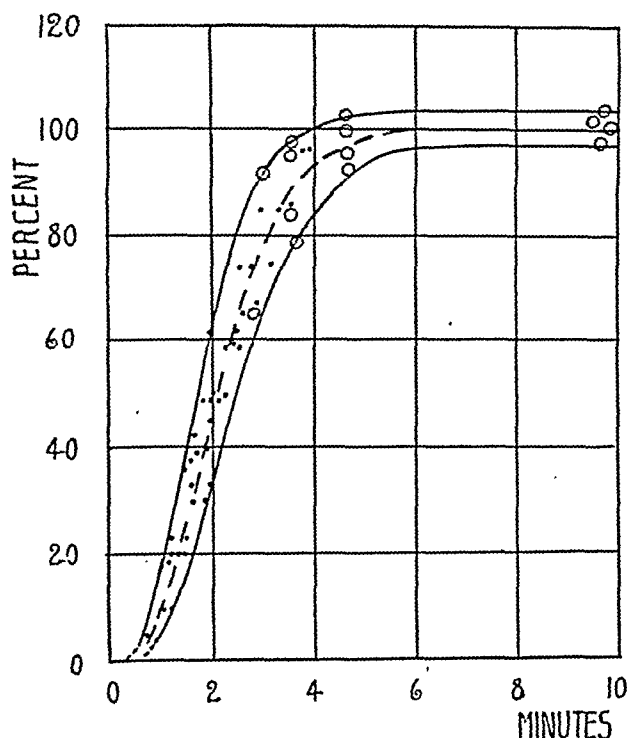


FIG. 6. The course of recovery of 5 cells after 0.5 second exposure to 1°C. The initial temperature was 35°C. and the measurements were made at 35°C. Coordinates as in Fig. 2.

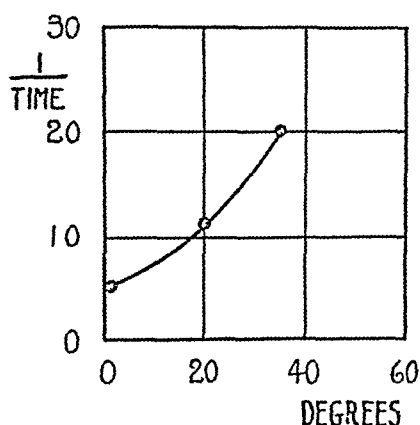


FIG. 7. The relation between time for complete recovery and the temperature maintained during recovery made. In all cases there was an exposure of 0.5 seconds to 1°C. The ordinates represent the time necessary to recover and the abscissae the temperature. See text.

recovery process, using a Q_{10} value. Fig. 6 shows the recovery from 0.5 second exposure to 1° when the preceding temperature was 35° . The recovery is much more rapid than at 20° and its duration may be taken at approximately 5 minutes. From Fig. 2 the duration of recovery with 20° is about 8 minutes. That with 1° may be considered 20 minutes, because it is at the end of this time that immediate resumption of streaming in 20° takes place. If we take the reciprocals of these times as representing the relative velocities and plot them against temperature we get Fig. 7. Using the Van't Hoff formula

$$Q_{10} = \left(\frac{K_2}{K_1} \right)^{\frac{10}{T_2 - T_1}}$$

we find a Q_{10} value of 1.51 between 1° and 20° and 1.45 between 20° and 35° . This coefficient is of the order usually expected of physical phenomena, and to this extent corroborates the idea already advanced that the inhibition and recovery of streaming, as occasioned by a sudden fall of temperature, have a physical basis inherent in the structure of protoplasm.

SUMMARY.

A sudden fall of temperature of 15° to 20° causes a complete cessation of the protoplasmic streaming in *Nitella*. The recovery of the normal rate follows a definite course and the time of recovery is dependent on the temperature. Evidence is adduced to show that the inhibition of streaming is due to a physical phenomenon.

The author wishes to extend his thanks to Professor A. R. Davis and Professor D. R. Hoagland for their kindness in supplying the *Nitella* used in these experiments.

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A GLASS ELECTRODE APPARATUS FOR MEASURING THE pH VALUES OF VERY SMALL VOLUMES OF SOLUTION.

By D. A. MACINNES AND MALCOLM DOLE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, May 11, 1929.)

In a recent article¹ the authors have described a new type of glass electrode, which is of convenient size, and at the same time of sufficiently low resistance for accurate work. Such an electrode is shown diagrammatically on the right-hand side of Fig. 1. The lower end of glass tube *A* supports the glass diaphragm *D* which in our experiments has been about 0.001 mm. thick. The tube *A* is partly filled with 0.1 N HCl into which is inserted the silver-silver chloride electrode *B*. More details concerning these electrodes are given in the article referred to above.²

Since glass electrodes of this type with glass diaphragms 4 mm. or less in diameter can be used, they seemed admirably adapted to measurements involving very small amounts of material. The need for such measurements arises frequently, especially in biological investigations. The apparatus shown diagrammatically in Fig. 1 was therefore designed to adapt the electrodes to this purpose. The vessel *C* holds a reference saturated calomel electrode. This electrode is connected, through a stopcock, with the reservoir *R* which contains

¹ MacInnes, D. A., and Dole, M., *J. Ind. Eng. Chem.* (Analytical Edition), 1929, i, 57. This paper contains references to the previous work with glass electrodes, with the exception of a recent article by Mirsky and Anson (Mirsky, A. E., and Anson, M. L., *J. Biol. Chem.*, 1929, lxxxi, 581.

² Through the kindness of Dr. Alexis Carrel we have tested the utility of these electrodes in connection with tissue culture work. The usual vessel for that work was provided with three outlet arms. One arm contained a tube through which the medium could be drawn to make contact with a saturated calomel electrode, and the other two arms held glass electrodes. One glass electrode was kept in the medium and the other in the immediate vicinity of the tissue studied. In this way it was possible to follow accurately the change of pH with time of the tissue and the medium separately.

a supply of saturated KCl solution. Another branch of the tubing connects with the tip *T*. An additional branch tube *F* is closed by a piece of rubber tubing (a so-called "policeman") on which a screw pinchcock is placed. The whole apparatus is mounted on adjustable screw clamps, as shown in Fig. 2, so that the glass electrode and the calomel electrode with its attachments can be independently raised and lowered. The clamp holding the glass electrode is insulated from the rest of the support with a piece of Bakelite.

To make a pH determination the following steps are necessary. The rubber tube on *F* is compressed by the pinchcock. The stopcock below the reservoir

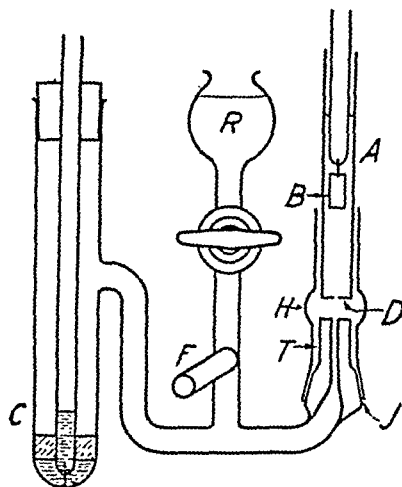


FIG. 1. Diagram of apparatus

R is then opened slightly and saturated KCl is run out on the tip *T*. With a piece of filter paper this solution is removed from the tip so that the solution lies in the capillary and flush with the surface. A drop of the solution whose pH is desired is then placed on the tip. By loosening the pinchcock on *F* slightly this drop is drawn into the capillary tube so that the liquid junction is lowered a few millimeters below the surface of the tip. The remainder of the drop is then removed with filter paper. This procedure has the effect of removing any KCl-bearing solution from the tip. Another drop of the solution under observation is then added, and the protecting tube *H* is put in place as shown. The glass electrode is next lowered, by means of the screw adjustment, until the glass diaphragm *D* comes in contact with the drop. An E.M.F. measurement may then be made with an electrometer and potentiometer.

It is necessary to put a thin coating of paraffin on the tubing up to the edge of

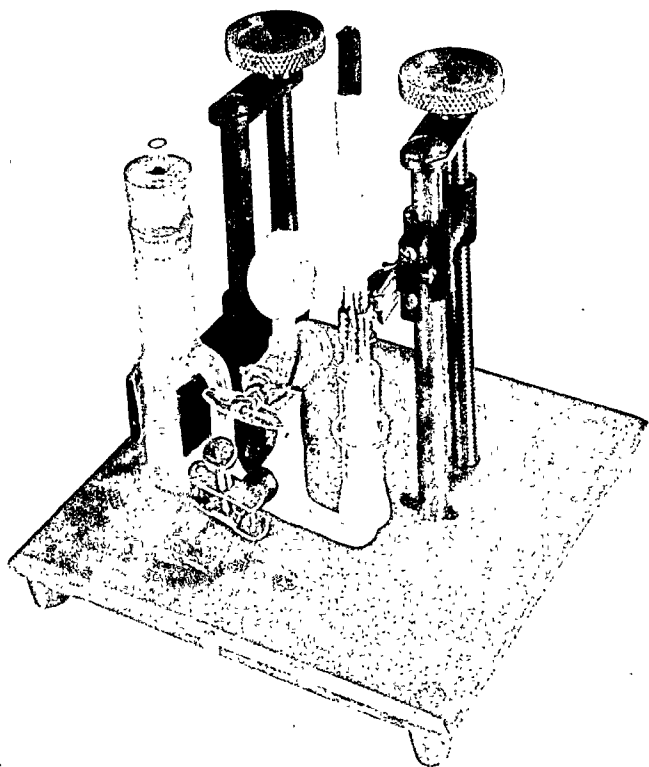


FIG. 2. Glass electrode apparatus for measuring the pH of very small volumes of solution

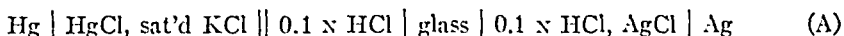
the tip T to prevent the drop of solution from spreading. A similar coating around the lower edge of the glass electrode is also desirable for the same reason. The protecting tube H was added to the apparatus after finding that the potentials observed were unsteady and drifting due to evaporation from the edge of the drop. It is important to have a close fitting ground joint at J so that rising air currents are prevented.

Our measurements have been made in a constant temperature room, at 25°, with a "Type K" Leeds and Northrup potentiometer, using a Compton electrometer, made by the Cambridge Scientific Instrument Co., as a null instrument. Readings could be made to about 0.2 millivolt. Due to the relatively low resistances of the glass electrodes (about 10 megohms in our most recent measurements) little screening of the electrical system was found necessary. For the same reason ordinary care in insulation was ample. The connections from the potentiometer to the electrometer were made with lead-screened wire.

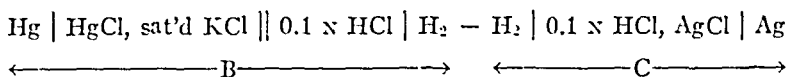
The formula to be used in computing pH values from the measurements at 25°C. with the apparatus as described is

$$\text{pH} = \frac{E + G + 0.1066}{0.05915}$$

in which E is the measured potential, and G the potential (to be discussed below) which may be present in the glass. The constant 0.1066 can be obtained as follows. The potential of the system



will be the same as the combination



if the glass acts as a hydrogen electrode and is the source of no other potential. The potential of the cell B is,³ at 25°,

$$E = 0.2458 - 0.05915 \log a_H$$

in which a_H is the activity of the hydrogen ion in 0.1 N HCl. The cell C has the potential -0.3524 .⁴ The total potential is therefore

$$E = 0.2458 - 0.3524 - 0.05915 \log a_H \quad (\text{D})$$

³ Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928, p 672.

⁴ From the work of Scatchard, G., *J. Am. Chem. Soc.*, 1925, xlvii, 641, involving a slight interpolation.

Now if we raise the hydrogen ion activity of the acid solution nearest the calomel electrode in the cell *A* to unity a potential will arise at the glass surface equal to

$$E = +0.05915 \log a_H \quad (E)$$

so that the new potential will be equal to the sum of *D* and *E* or

$$E_0 = 0.2458 - 0.3524 = -0.1066$$

which is the constant in the equation

$$E = E_0 - 0.05915 \log a_H = E_0 + 0.05915 \text{ pH}$$

or the potential of the cell *A* when the hydrogen ion activity between the glass and the saturated KCl solution is unity.

The potential *G* in the glass may be determined by placing the glass electrode (with the solution and silver-silver chloride electrode) in a beaker containing 0.1 *N* HCl and another silver-silver chloride electrode. In our more recent work we have used glass of a composition which gives membranes in which this potential is nearly zero. This and other information we have obtained concerning the relation of the composition of glass to its behavior when made into electrodes will be published elsewhere.

A number of tests were made to see whether the apparatus as described would yield correct pH values. For this purpose four glass electrodes were used with a buffer solution of pH 7.76, as determined by the hydrogen electrode. The results are shown in Table I.

TABLE I.

Electrode	Date	pH
1	Apr. 24	7.84
2	" 24	7.74
3	" 24	7.77
3	" 24	7.75
3	" 25	7.76
4	" 25	7.76
1	" 25	7.76
1	May 2	7.74

Except for Electrode 1 (which initially gave an error of 0.08 pH unit) the measurements are all within 0.02 unit (about 1.2 millivolts).

Extensive tests (not made with this apparatus) have shown that

electrodes, made with the glass we have found most suitable, begin to deviate 0.02 pH unit from the correct values at pH 9.5 and show rapidly increasing deviations at higher pH values, if the solution measured is 0.1 N in sodium ion. The deviations begin at lower pH values if the sodium ion concentration is greater. In alkaline solutions the potentials may be dependent on the nature of the positive ions present and may vary with time. A more complete description of these tests will appear in another article.

An opportunity for testing the usefulness of this apparatus arose in connection with the work of Dr. Marian Irwin of this Institute. An important question to be investigated was whether the penetration of a basic dye changes the pH value of the vacuolar sap of living cells of *Nitella*. A few drops only of this sap can be conveniently obtained at one time. The question could not be settled by means of the hydrogen electrode since it is "poisoned" by the sap, and by the dye, brilliant cresyl blue, which was used. Such poisonings, which are, in many cases at least, due to irreversible oxidation-reduction potentials, do not appear to have any effect on the glass electrode.⁵ Furthermore, the use of hydrogen gas would affect the concentration of CO₂ on which the pH value partly depends. The use of indicators with these dye solutions is obviously impossible. By employing the apparatus described above on samples of the sap prepared by Dr. Irwin it was shown that the entrance of the dye raises its pH value considerably. The results of these experiments will be fully described elsewhere.

⁵ So far as we have been able to test the glass electrodes they seem, up at least to pH 9, in dilute salt solutions, to react only to changes in hydrogen ion activity. For instance, the paper referred to (1) gives data on the electrometric titration of sulfuric acid in the presence of potassium permanganate. The potentials followed the course to be expected from the change of hydrogen ion activity and were uninfluenced by the strong oxidation potential of the permanganate. On the other hand, the glass electrodes showed no change in potential when ferrous sulfate was titrated with potassium dichromate in the presence of an excess of sulfuric acid. In this case the hydrogen ion concentration remained substantially constant during the titration although there was a change in an oxidation-reduction potential of roughly 0.3 volt. We have other evidence, bearing on this matter, which cannot be conveniently summarized and will be published later.

SUMMARY.

A glass electrode apparatus is described with which pH measurements can be made with as small volumes as 2 drops (about 0.14 cc.) of solution.

Using this apparatus the change of pH of the vacuolar sap of *Nitella*, due to the penetration of brilliant cresyl blue, has been readily followed. The sap and the dye have been found to poison the usual type of hydrogen electrode.

THE VALIDITY OF THE GLASS ELECTRODE IN AMMONIUM CHLORIDE BUFFERS.

By SAMUEL E. HILL.*

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(Accepted for publication, March 25, 1929.)

In the course of experiments on the cytolysis of luminous bacteria it became necessary to know accurately the pH values of 0.5 M solutions of ammonium salts from about pH 5.0 to pH 9.6. Partly because of the high salt concentration colorimetric methods were insufficiently accurate, especially on the alkaline side. On account of the oxidation of quinhydrone in alkaline solutions the quinhydrone electrode could not be applied to more than one-half of the range of values desired. The statements in the literature as to the action of ammonia as a hydrogen electrode poison are conflicting,¹ and also the hydrogen electrode is too slow to be used conveniently when the number of determinations may exceed 100 in a single experiment. The glass electrode seemed to offer the best solution to the problem, being free of salt errors and of all other errors due to changes in the solution as a result of the examination itself. It is said to be accurate to 0.01 pH.² The statements as to the accuracy of the glass electrode in alkaline solutions vary. Brown³ reports good agreement with the formula $E = 0.058 \log (C_1/C_2)$ from pH 4.0 to pH 10.0. Hughes⁴ reports approximate agreement with the hydrogen electrode below pH 11.0 and in another paper⁵ states that the electrode values were unaffected by large changes in sodium ion concentration below pH 9.0. Since the writer has occasion to use ammonium solutions above pH 9.0 both in presence and absence of sodium chloride, the

* Fellow in Zoology of National Research Council.

¹ Cf. Clark, The determination of hydrogen ions, 3rd ed., 1928, p. 439.

² Kerridge, P. M. T., *J. Sci. Ins.*, 1926, iii, 404.

³ Brown, W. E. L., *J. Sci. Ins.*, 1924, ii, 12.

⁴ Hughes, W. S., *J. Am. Chem. Soc.*, 1922, xlv, 2860.

⁵ Hughes, W. S., *J. Am. Chem. Soc.*, Feb., 1928, p. 491.

experiments described herein were made to determine the validity of the glass electrode in the class of solutions employed. Since others also may be interested in the use of fairly accurately determined ammonium buffers of relatively high concentration, these experiments are recorded here.

In the dry air of the winter laboratory sulfur was found an excellent insulating material and no trouble was experienced from leakage. The

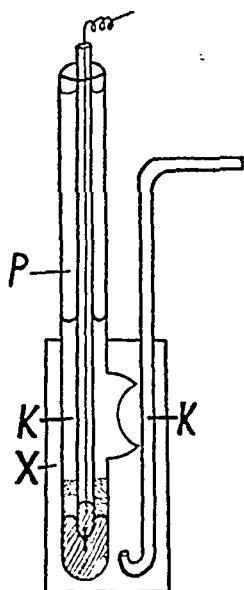


FIG. 1. Glass electrode vessel. K = Saturated KCl. P = Paraffine oil. X = Unknown solution.

form of electrode used is a combination of those of Hughes⁴ and Kerridge,⁶ a modification suggested by Anson and Mirsky,⁷ and further slightly modified by the writer. In the form finally adopted, illustrated in Fig. 1, the delicate glass membrane is protected from the breakage which is inevitable when solutions are pipetted in and out of the cup as suggested by Kerridge,⁶ and is to be preferred when 15 or 20 cc. of solution is available for the test. A saturated calomel

⁶ Kerridge, P. M. T., *Biochem. J.*, 1925, xix, 611.

⁷ Anson, M. L., and Mirsky, A. E., *Proc. Soc. Exp. Biol. and Med.*, 1928, xxv, 346.

electrode is made in the lower part of the glass electrode vessel and the saturated KCl in contact with the glass membrane is standardized daily against $m/20$ KH phthalate. All calculations are made from its pH of 3.97 by the formula

$$\text{pH}_x = 3.97 \pm \frac{E_s - E_x}{0.0581}, \text{ at } 20^\circ,$$

where E_s = p.d. of KH phthalate,

E_x = p.d. of unknown.

A Lindeman electrometer is used as the null instrument.

In the first experiment, 0.5 m ammonium chloride plus 0.5 m ammonia was diluted with successive equal portions of 0.5 m am-

TABLE I.

Activity Coefficients of Several Concentrations of Ammonium Chloride.

f_{a1} is the empirical value, f_{a2} that calculated from Bjerrum's formula.

[NH ₄ Cl]	[NH ₃]	pH	f_{a1}	f_{a2}
0.01	0.00207	8.65	0.8335	0.8532
0.1	0.0207	8.70	0.7437	0.7104
0.25	0.05075	8.73	0.6804	0.6286
0.5	0.1015	8.75	0.6502	0.5564

monium chloride until the concentration of ammonia approached 0.001 m . A similar series was prepared in which 0.5 m ammonium chloride plus 0.5 m sodium chloride plus 0.5 m ammonia was diluted with 0.5 m ammonium chloride plus 0.5 m sodium chloride. The pH values of these solutions were taken and plotted against the logarithm of the concentration of ammonia (Fig. 2). For the ammonia plus ammonium chloride series these values fall on a straight line within the experimental error of the electrode, showing that there is no error due to any change in the electrode at the higher concentrations of hydroxyl ions. For the ammonia plus ammonium chloride plus sodium chloride series the values fall on the same straight line until about pH 8.6, the deviation from this point increasing until there is an error of -0.07 pH at pH 9.4. Considering the experimental error of the method (0.01 pH), corrections are unnecessary below pH 8.6 in the

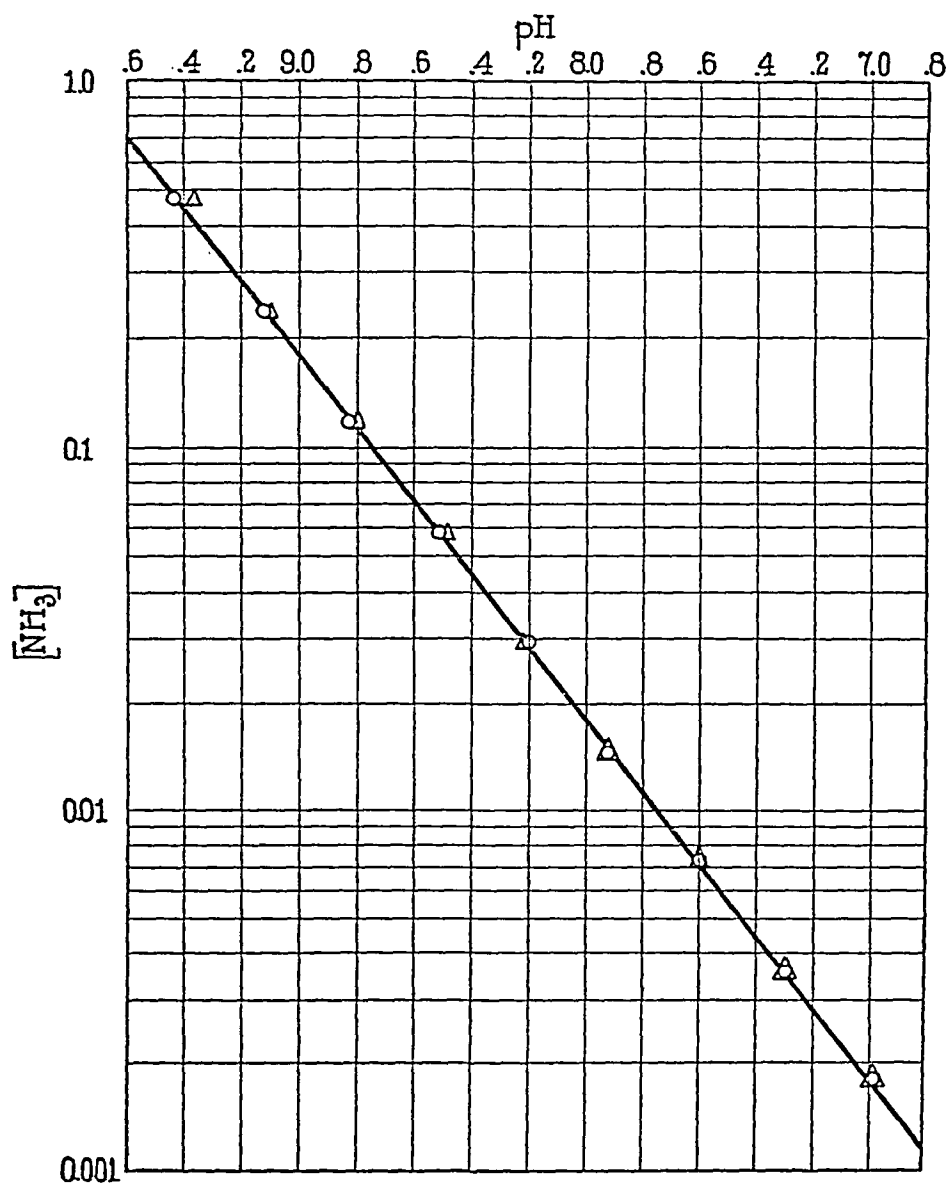


FIG. 2. O = pH of indicated concentration of ammonia in presence of 0.5 M ammonium chloride. Δ = pH of indicated concentration of ammonia in presence of 0.5 M ammonium chloride plus 0.5 M sodium chloride.

series containing sodium chloride and unnecessary at any value as far as determined in the absence of sodium ions (and perhaps the ions of the other alkali metals). For ammonium chloride solutions in presence of 0.5 M sodium chloride the corrections given in Table II are suggested.

In the physiological study in question, the relation of concentration of ammonia to concentration of hydrogen ions is of considerable importance and can be calculated readily if the activity coefficients of the ammonium salts are known. The formulae for calculating these values accurately are of such an involved nature as to be useless for

TABLE II.

Apparent pH of Ammonium Chloride Buffers 0.5 M With Respect to Ammonium Chloride in Presence of 0.5 M Sodium Chloride, With Corrections.

pH	Correction
8.6	+ .01
8.8	+ .02
9.0	+ .03
9.2	+ .04
9.4	+ .07

the average worker in the biological laboratory.⁸ The activity coefficient may be determined empirically from the pH values and the concentrations of ammonia and ammonium chloride. Choosing for an example the sample containing 5.8×10^{-2} mols ammonia per liter, pH 8.51, which happens to give the average value for the series, and applying the buffer equation:

$$[\text{OH}^-] = \frac{K_b [\text{NH}_3]}{f_a [\text{NH}_4]}$$

$$f_a = \frac{1.8 \times 10^{-5} \times 5.8 \times 10^{-2}}{3.24 \times 10^{-6} \times 0.5} = 0.6444$$

The empirical values were determined for several concentrations of ammonium chloride and these are compared in Table I with the

⁸ Cf. Lewis, G. N., and Randall, M., *J. Am. Chem. Soc.*, 1921, xliii, 1112.

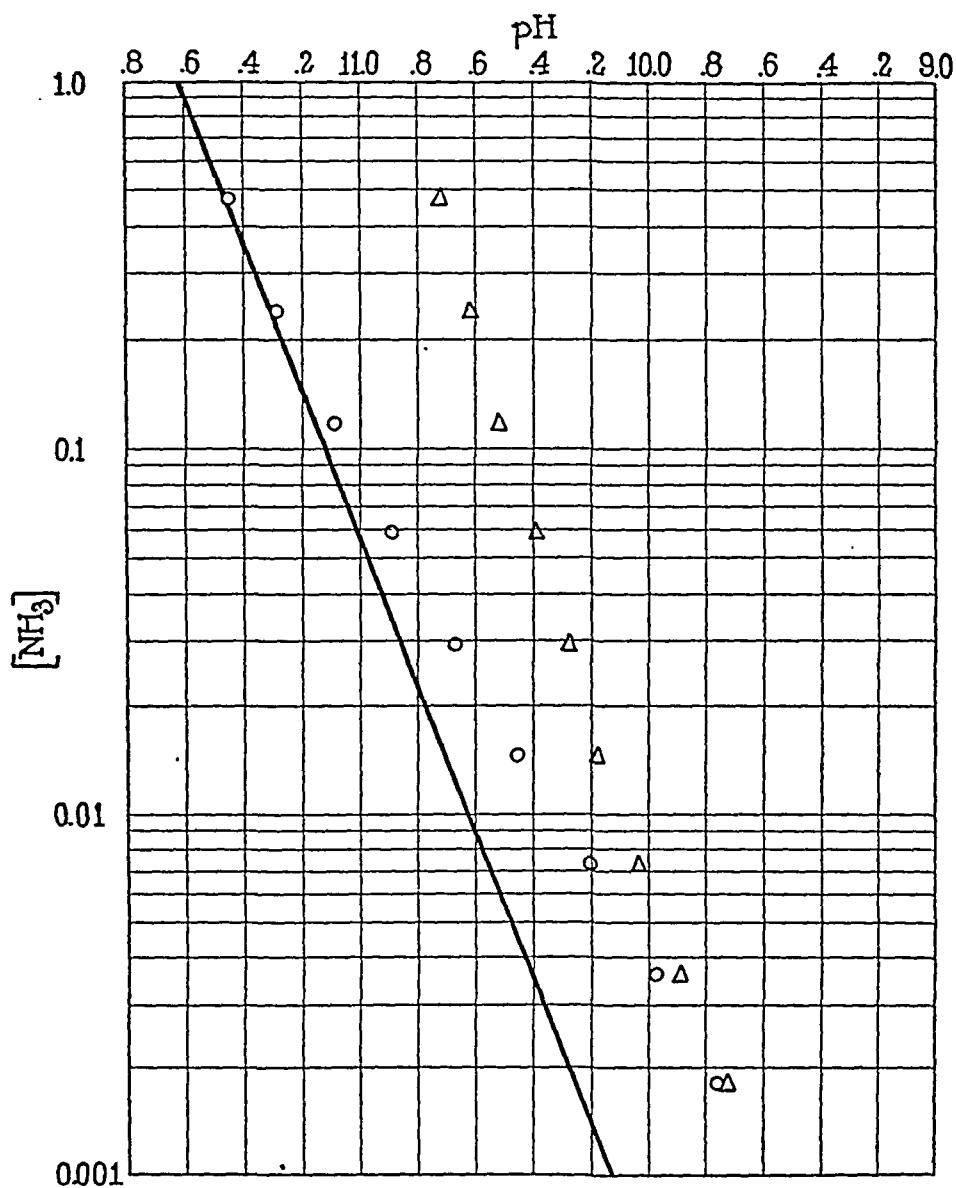


FIG. 3. Comparison of theoretical pH of ammonia in water with that observed with the glass electrode. O = Ammonia in water. Δ = Ammonia in 0.5 M sodium chloride solution.

calculated values from the simplified approximation formula of Bjerrum,⁹ said not to be valid above about 0.1 M.

$$-\log f_a = \frac{26}{D} n^2 \sqrt[3]{c};$$

D is the dielectric constant of the solvent, and n the valence of the ions. As may be seen by reference to the table the agreement is not remarkable at any concentration, but is much better for 0.01 M and 0.1 M than for 0.25 M and 0.5 M solutions. The writer is inclined to think the empirical value for 0.5 M ammonium chloride the more accurate, and will use it in his calculations.

The measurement of pH of ammonia in water and in 0.5 M sodium chloride was less satisfactory. In the biological work involved, exclusion of carbon dioxide is not possible, so it was not attempted in this case. At the lower concentrations of ammonia the carbon dioxide introduced a serious error by the formation of an ammonium bicarbonate buffer system. At high concentrations of ammonia where the error due to carbon dioxide is negligible, there was no significant deviation from the calculated values in pure water, while in 0.5 M sodium chloride there was a large negative variation, 0.74 pH at pH 11.46. This variation decreased progressively as the ammonia was diluted and the manner of approach of the curves suggests its disappearance at about pH 9.0. For ammonia solutions from which sodium chloride is absent, the glass electrode may be used without appreciable error to pH 11.4 and perhaps higher. If sodium chloride is present a correction must be applied above pH 8.6, and will probably have to be determined anew for each electrode by the use of appropriate buffer solutions in which sodium ions are present in approximately the same concentration as in the unknown solution.

SUMMARY.

It is shown that the glass electrode may be used without appreciable error to measure pH of ammonia or ammonium chloride buffers, but that corrections must be applied above pH 8.6 if sodium ions are present in the unknown solution. Corrections are given for values from pH 8.6 to 9.4. A slight further modification of form of glass electrode used by previous workers is described.

⁹ Cf. Michaelis, Hydrogen ion concentration, p. 121.

A METHOD FOR THE TITRATION OF COMPLEMENT.

By HARRY EAGLE.

(From the Department of Pathology and Bacteriology, Johns Hopkins Medical School, Baltimore.)

(Accepted for publication, March 16, 1929.)

A major difficulty in the quantitative study of complement is the accurate determination of the amount remaining after various manipulations. The method usually employed is to compare the minimal quantity of solution which will completely hemolyze a measured volume of sensitized cells with the corresponding quantity of the original serum. The method is cumbersome and time-consuming, necessitating multiple tests for each determination, and the results are only approximate.

Recently, Brooks¹ has suggested a method based on the colorimetric estimation of the proportion of cells hemolyzed by a given quantity of solution within an arbitrarily fixed time. By the use of a curve correlating the percentage of hemolysis with the quantity of complement he obtains results accurate to 1 per cent.

Needing a simple but accurate method adapted to making many determinations within a relatively short time, we have made use of the fact that the time required for hemolysis is a function of the quantity of complement. The data for a reference curve similar to Fig. 1 are first obtained with known quantities of complement, using a fixed amount of a standard sensitized cell suspension. The time required for an unknown solution to cause hemolysis *under the same conditions of temperature and volume* is then determined, and its complement content obtained by graphic interpolation on the time-complement curve (Fig. 1).²

¹ Brooks, S. C., *J. Med. Research*, 1919-20, xli, 399.

² Strictly speaking, the term minimal hemolytic quantity (unit) of complement has no significance unless qualified by the conditions under which the readings are made. In the experiment here reproduced, the unit of complement at 5 minutes is 0.026 cc., at 10 minutes, 0.0085 cc., and at 60 minutes < 0.004 cc. If it were not for an increasingly more rapid inactivation of complement as it is diluted, an infinitesimal quantity would suffice to cause hemolysis.

The following are the variables which must be controlled, and the values found most convenient:

Temperature.—37°C. If all determinations are made within an hour, it is possible to work at room temperature.

Final Volume.—2 cc.

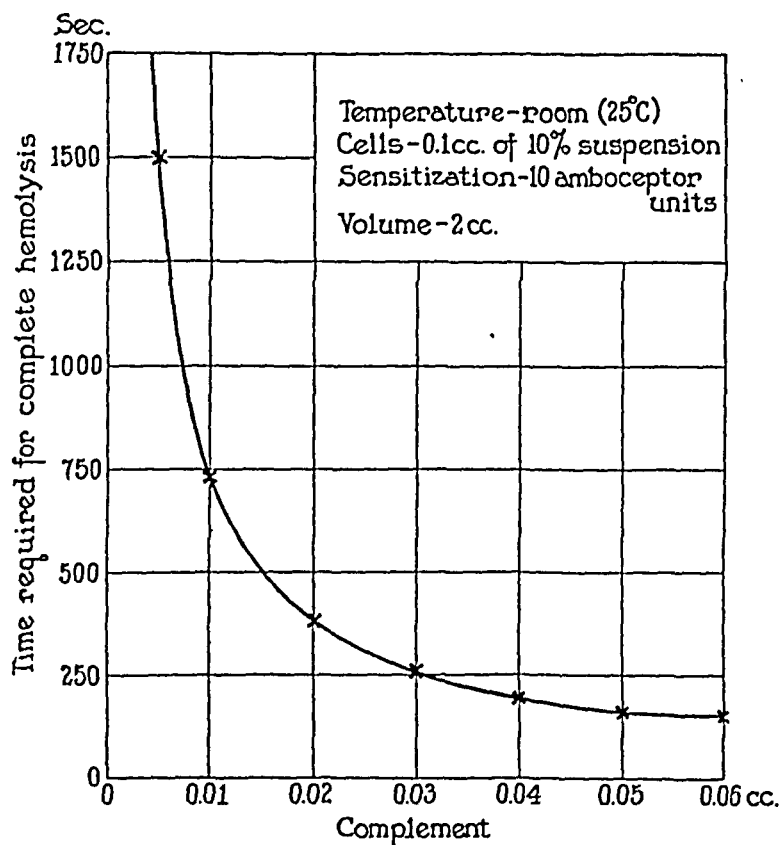


FIG. 1. Effect of quantity of complement upon the time required for complete hemolysis.

† *Test Suspension of Sensitized Cells.*—X cc. of sheep's blood, citrated, are washed twice in 10 volumes of 0.85 per cent NaCl (at pH 7.4), and the sedimented cells resuspended to 10 times the original blood volume in NaCl. There are then added 15 sensitizing units of an immune rabbit serum. After 1 hour at room temperature ($\frac{1}{2}$ hour at 37°) the suspension is ready for use.

Order of Addition.—X cc. of the unknown solution (or of complement in obtaining data for the reference curve) are made up to a total volume of 1.9 cc. with

0.85 per cent NaCl, and the solution poured into a Wassermann tube containing 0.1 cc. of the sensitized cell suspension. The time required for *complete* hemolysis is noted, and the complement content determined in the manner described.

Provided only that the same test-cell suspension is used, under the same conditions, as many as 20 determinations can be made in an hour, 5 to 10 simultaneously.

It is obvious that any error in reading the endpoint is minimized in the steep descending portion of the curve, and exaggerated in the horizontal portion. Where feasible, it is therefore advisable to use that quantity of unknown solution which will give a hemolysis time in the zone of least experimental error.

THE MECHANISM OF COMPLEMENT FIXATION.

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I.

Complement Fixation a Secondary Process in All the In Vitro Immune Reactions.

The *in vitro* immune reactions may be tabulated as follows:

- | | | |
|---|-----------------------------|--------------------------------------|
| 1. Bacteria | + Immune serum | → Agglutination |
| 2. Soluble protein antigen
(sheep serum, egg
albumen, etc.) | + Immune serum | → Precipitation |
| 3. Red cells | + Immune serum (amboceptor) | → Sensitization
(± Agglutination) |

It was shown by Bordet and Gengou (5) that the first of these reactions is accompanied by complement fixation, *i.e.*, if fresh guinea pig serum is present, it loses its ability to hemolyze sensitized red cells. Shortly thereafter, it was shown (16) that a similar inactivation of complement is obtained during the second of these reactions and, indeed, is a more sensitive indicator of the antigen-antibody interaction than the visible flocculation.

Whether a similar fixation occurs during the sensitization of red cells is as yet undetermined, due, of course, to the fact that hemolysis takes place. Thus, Liefmann and Cohn (20) state that although it is true that complement disappears, this inactivation is almost entirely a *result* of hemolysis, due to as yet unknown complicating factors, rather than a fixation by the sensitized cells. And although Michaelis and Skwinsky (22) showed that at an acid reaction (pH 5.3) which inhibits hemolysis, there is fixation (of the midpiece fraction), the experiments have been objected to on the ground that the high degree of sensitization necessary (25 amboceptor units) and the acid reaction are not duplicated under normal conditions.

The use of a simplified quantitative method of complement titration has made it possible to establish quite definitely that sensitized cells do fix complement; that there is a complete analogy to complement fixation by either agglutinated bacteria or the immune precipitate; and that this fixation, far from being a result of hemolysis, precedes it.

Methods.

1. Fixators.

(a) Sensitized cells.—Citrate sheep's blood is washed twice in 10 volumes of saline (NaCl $N/7$), resuspended to 10 times its original volume, and sensitized with 10 units of a rabbit anti-sheep serum.

(b) Immune precipitate.—Sheep plasma is incubated with the optimum quantity of a rabbit anti-sheep serum in 10 volumes of NaCl. The resulting precipitate, consisting almost entirely of globulins of the immune-serum, is washed twice in 10 volumes of NaCl, and resuspended to 15 times its original volume. As little of 0.1 cc. of this suspension, representing 0.02 cc. of the serum used, fixes 0.4 cc. complement (20 units) almost completely.

(c) Agglutinated bacteria.—*B. typhosus*, washed free of the broth medium, incubated with a rabbit anti-serum. The agglutinated bacteria, washed 3 times in 10 volumes of NaCl, are resuspended to 15 times their own volume in NaCl.

2. Complement.—Guinea pig serum.

3. Titration of residual complement.

The method used is described in detail elsewhere (12), and is based on the fact that the time required to hemolyze a fixed volume of a standard sensitized cell suspension, is, under constant conditions of temperature and volume, a function of the quantity of complement. For the determinations, the fluid, freed from the fixing substance by strong centrifugation, is poured into a tube containing 0.2 cc. of a 10 per cent sheep cell suspension sensitized with 10 units of a hemolytic serum, and the time required for complete hemolysis noted.

Protocol 1. The Adsorption of Complement by Sensitized Cells.—To a series of tubes containing X cc. of sensitized cells are added 0.04 cc. of complement and NaCl ($N/7$) to 2 cc., at varying times before beginning of centrifugation. The tubes are placed in the centrifuge as they are prepared, and a minimum time allowed to elapse between the preparation of last tube and beginning of centrifugation (2500 r.p.m. for 1 minute). The clear supernatant fluid is poured into a tube containing 0.1 cc. of sensitized cells, and the time necessary for complete hemolysis noted. By a method already described (12), the amount of complement present in the supernatant may be calculated from the velocity of hemolysis. This quantity subtracted from that originally present, gives that adsorbed by the sensitized cells.

The time X in the figures corresponds to the adsorption which takes place

during centrifugation. The sedimentation of the strongly sensitized cells is so rapid that X is certainly less than 20 seconds, and in any one experiment, is obviously the same in all the tubes.

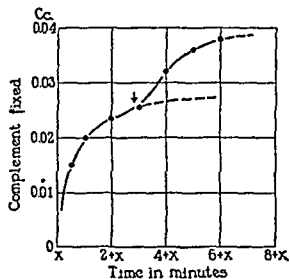


FIG. 1.

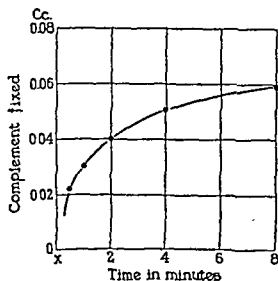


FIG. 2.

FIG. 1. Complement fixation by suspension of sensitized cells.

FIG. 2. Complement fixation by the precipitate formed in an antigen-antibody reaction.

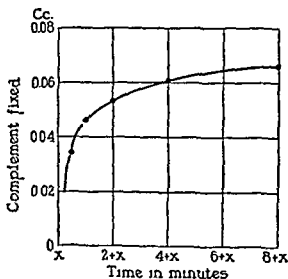


FIG. 3. Complement fixation by a suspension of agglutinated bacteria.

The inflection in the curve, signifying a sudden increase of fixation, is coincident with the beginning of hemolysis and explains the experimental data of Liefmann and Cohn. Its significance will be discussed

in a later paper. The dotted lines indicate the probable course if it were not for this confusing secondary fixation.

For comparison, there are introduced similar curves for the velocity of complement fixation by agglutinated bacteria, and by the globulin suspension. In Fig. 1, the arrow indicates *beginning* hemolysis, as shown by the tint of hemoglobin in the supernatant fluid. Obviously, fixation precedes hemolysis. Indeed, at low temperatures, where the velocity of hemolysis is very greatly retarded, it is possible to obtain a complete dissociation of the two reactions. (See Temperature Coefficient of Fixation.)

We may therefore complete the diagrammatic tabulation of the *in vitro* immune reactions as follows:

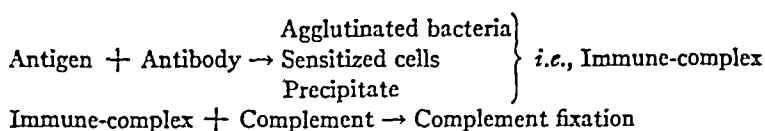
- | | |
|--|--|
| 1. Agglutinated bacteria | + Complement → Complement fixation ± Bacteriolysis |
| 2. Sensitized red cells | + Complement → Complement fixation ± Hemolysis |
| 3. Immune precipitate | + Complement → Complement fixation ± Anaphylatoxin |
| 4. Beef heart extract-syphilitic serum complex | + Complement → Complement fixation |

Although most observers have concerned themselves with only the last two of these reactions, even a cursory survey of the literature relating to the theories of complement fixation would go far beyond the scope of the present paper (17), (27), (30). The recent tendency has been to regard Reactions 3 and 4 as a non-specific physical process, in which the aggregates formed during the antecedent antigen-antibody reaction adsorb complement, *i.e.*, a labile component of fresh serum which has the property of hemolyzing sensitized cells.

The following experiments are in full accord with such a hypothesis, and extend it to include all of the *in vitro* immune reactions listed above.

II.

The Kinetics of Complement Fixation.



It is apparent that in order to arrive at the kinetics of the second reaction, it is essential to eliminate the time and quantity factors introduced by the first. Such a reaction as the Wassermann, in which

antigen, "antibody" and complement are added simultaneously, is of no value in arriving at the physical constants of complement fixation. No matter how exactly measured, the results obtained apply to neither of the two reactions individually, but to a complex of two consecutive reactions of quite different character. For this reason, in the following experiments the fixing agent used was an antigen-antibody mixture which had been allowed to come to equilibrium before the addition of complement.

A. Effect of Reactants upon the Velocity of Fixation.

Protocol 2.—To X cc. of the standard globulin suspension are added 1.0 cc. of 1/10 complement at the times noted in Table I, the tubes being placed in centrifuge

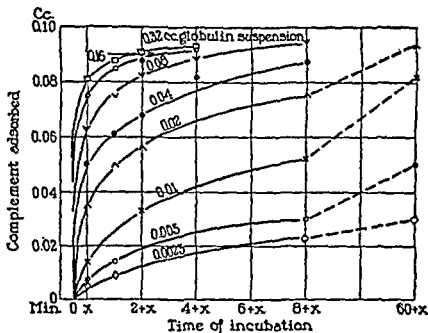


FIG. 4. Effect of globulin concentration upon velocity and degree of complement fixation.

cups as they are prepared. At the indicated time, centrifugation is begun, 60 seconds at 2500 r.p.m. sufficing to throw down practically all the particles of denatured globulin.

The supernatant fluid is poured directly into a tube containing the test cells (see page 826) and the time required for hemolysis noted. The quantity of free complement corresponding to this hemolysis time, subtracted from the original quantity added, gives that fixed by the globulin suspension (Fig. 4).

In the curves, the time X corresponds to the fixation which occurs during cen-

trifugation before the globulin is completely sedimented. In any one experiment, this is obviously the same for all the tubes, and is probably less than 20 seconds.

If now, the quantity of globulin is kept constant, and that of complement varied, we obtain the series of curves given in Fig. 5.

The essential identity of all the fixation reactions is again illustrated by the fact if one uses sensitized cells or agglutinated bacteria as the fixing agent instead of the globulin suspension, exactly similar sets of curves are obtained (Figs. 6, 7, 8).

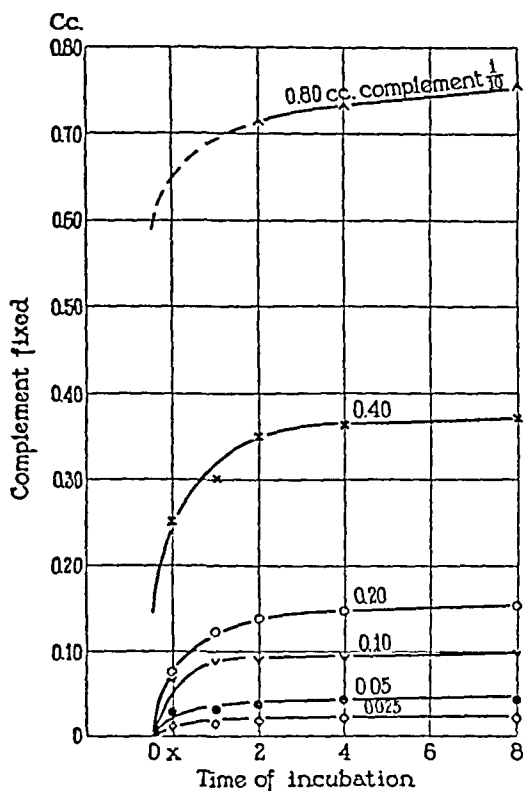


FIG. 5. Effect of (complement) upon velocity and degree of complement fixation by globulin suspension.

In all the experiments, increases of either of the two reactants has the same effect: more complement is fixed, and the reaction accelerated. *Qualitatively*, therefore, the reaction proceeds according to the mass action law.

$$\frac{[\text{Complement}] \times [\text{Antigen-antibody complex}]}{[\text{Complement fixed, i.e., Antigen-antibody-complement}]} = K$$

and the velocity of reaction

$$\propto [\text{Complement}]$$

$$\propto [\text{Antigen-antibody complex}]$$

The steep initial rise, and the rapid change in slope as the equilibrium value is approached, are difficult to interpret; but it is quite significant that similar curves have been obtained for the most diverse kinds of

TABLE I.

Data Used in Plotting Curve 0.02 of Fig. 4.

Time of addition of globulin to complement	Minutes before centrifugation	Time at which supernatant is added to test cells	Time at which hemolysis of test cells is complete	Seconds required for complete hemolysis	Complement remaining free in solution	Complement adsorbed by globulin
					cc.	cc.
10:15	60 + x	11:18:50	11:41:50	1380	0.006	0.094
11:07	8 + x	11:18:45	11:23:15	270	0.024	0.076
11:11	4 + x	Tube broken				
11:13	2 + x	11:18:35	11:21:50	195	0.043	0.057
11:14	1 + x	11:18:30	11:21:20	170	0.05	0.05
11:15	x	11:18:25	11:20:55	150	0.063	0.037

Controls* with known quantities of complement.

Complement					
cc.					
0.0062	11:18:20	11:40:15	1320		
0.0125	11:18:15	11:27:35	560		
0.025	11:18:10	11:22:30	260		
0.05	11:18:05	11:20:55	170		
0.1	11:18	11:19:40	100		

* By plotting the time required for increasing quantities of complement to cause hemolysis (control tubes) one obtains the reference curve used in arriving at the values for residual complement given in Column 6. These control tubes must be duplicated with each similar experiment, using the same cell suspension under the same conditions.

adsorption processes, with a similar acceleration produced by increasing the concentration of either the adsorbent or adsorbate (2), (4), (21).

B. Temperature Coefficient.

Using a constant quantity of fixing suspension and complement, the

velocity of fixation was determined at various temperatures between 5 and 40°C. The results of three such experiments are summarized in Figs. 9, 10, 11, using the globulin suspension, agglutinated bacteria, and sensitized cells respectively, as the fixing agent.

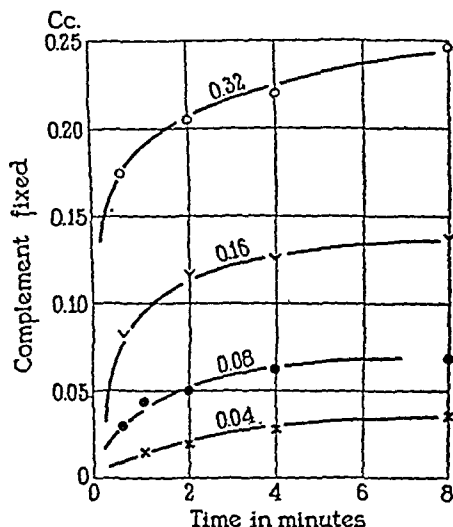


FIG. 6. Effect of (complement) upon complement fixation by sensitized bacteria.

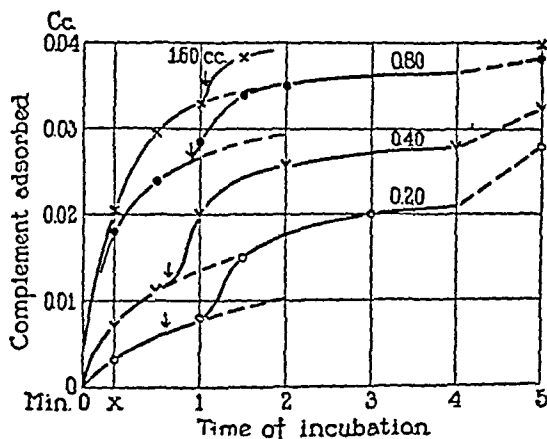


FIG. 7. Effect of (sensitized cells) upon velocity and degree of complement adsorption.

The similarity of the curves is further evidence of the essential identity of all the complement fixation reactions. It is apparent that the absolute magnitude of the temperature coefficient will depend entirely on its definition: if the amount fixed at equilibrium is taken as the

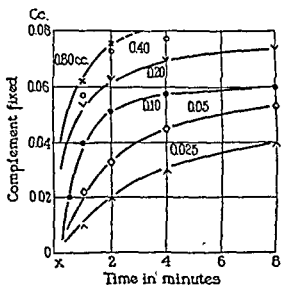


FIG. 8.

FIG. 8. Effect of (bacteria) upon complement fixation by a suspension of agglutinated bacteria.

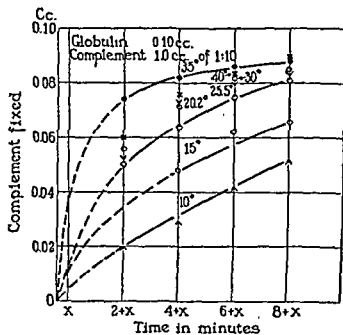


FIG. 9.

FIG. 9. Effect of temperature upon complement fixation by globulin suspension.

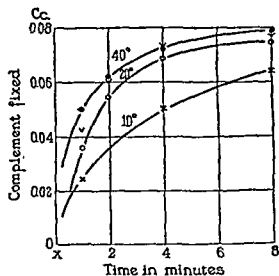


FIG. 10.

FIG. 10. Effect of temperature upon complement fixation by agglutinated bacteria.

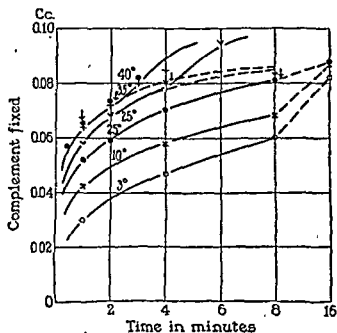


FIG. 11.

FIG. 11. Effect of temperature upon complement fixation by sensitized cells.

criterion, the coefficient is <1.1 , between 5 and 40°C.; if the velocities of fixation are compared, the value of the coefficient decreases steadily with the lapse of time, as shown in Table II. But, however expressed, the temperature coefficient is very low, averaging less than 1.3 between 10 and 40°C.

Although in itself not conclusive, such a low value suggests a surface

TABLE II.

Temperature Coefficient of Complement Fixation.

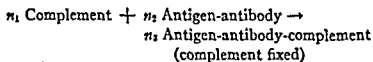
a = Globulin, *b* = Sensitized cells, *c* = Agglutinated bacteria.

Minutes	5°-10° (per 10)	10°-20°	20°-30°	30°-40°	10°-40° (per 10)
a					
1 b	2.5	1.3	1.3	1.05	$\sqrt[3]{2.2} = 1.30$
c		1.5			$\sqrt[3]{2.1} = 1.28$
a		2.25	1.12	1.07	$\sqrt[3]{3.3} = 1.49$
2 b	2.4	1.3	1.1	1.06	$\sqrt[3]{1.9} = 1.24$
c		1.5	1.17	1.05	$\sqrt[3]{1.8} = 1.22$
a		2.1	1.13	1.05	$\sqrt[3]{2.7} = 1.39$
4 b	2.3	1.28			$\sqrt[3]{1.6} = 1.19$
c		1.36	1.1	1.05	$\sqrt[3]{1.4} = 1.12$
a		1.6	1.06	1.05	$\sqrt[3]{1.8} = 1.22$
8 b	2.2				
c		1.16		1.05	$\sqrt[3]{1.25} = 1.08$
a					
16 b	<1.05	<1.05			<1.05
c					

reaction (adsorption) rather than a chemical reaction in the restricted sense of the term.

C. Quantitative Relationships in Complement Fixation.

If, varying the quantities of complement and fixing suspension, we determine, not, as heretofore, the velocity, but the conditions at equilibrium, we obtain results similar to those summarized in Table III, in which each horizontal row represents an individual experiment. Obviously, there is no stoichiometric relationship in the sense that



Nor does the degree of fixation correspond to a distribution of complement between fixing agent and solution in the ratio of its solubility in the two phases.

Qualitatively, however, the results do conform to the empirical equation of Freundlich for adsorption from dilute solutions: $\frac{x}{m} = Kc^{\frac{1}{n}}$

TABLE III.

Quantitative Relationships in Complement Fixation by I Globulin, II Agglutinated Bacteria.

To X cc. of (I) globulin suspension, (II) agglutinated bacteria, are added Y cc. of complement, and the total volume brought to 2 cc. with NaCl $N/7$. The figures given in the body of the table represent the complement fixed after 2 hours at room temperature (25°C.).

	Complement cc.	Fixing suspension								
		0.64 cc.	0.32 cc.	0.16 cc.	0.08 cc.	0.04 cc.	0.02 cc.	0.1 cc.	0.05 cc.	0.025 cc.
I	1. 1.28	1.273	1.26	1.24	1.12	0.96				
	2. 0.32			0.315	0.309	0.293	0.24			
	3. 0.16				0.157	0.1535	0.142	0.1		
	4. 0.08						0.077	0.071	0.05	0.028
II	5. 1.28	1.26	1.224	1.16	0.96	0.58				
	6. 0.64		0.623	0.6	0.534	0.44				
	7. 0.32				0.305	0.289	0.252	0.16		

where X is the total quantity adsorbed, m is the quantity of adsorbent, c is the amount of adsorbate remaining free at equilibrium, and K and n are constants, characteristic for each reaction, n always being greater than unity. It is seen from Fig. 12 that both types of fixation follow such a curve very closely. The n value of all but one of the curves fitted to the experiments in Table III is very close to 1.5.

If the Freundlich equation were an exact expression of the reaction, then the logarithms of $\frac{x}{m}$ plotted against the logarithms of c , would

fall along a straight line, whose slope is determined by the value of n . It has been shown for many types of adsorption that this is true only at low values of c ; that as the amount of free adsorbate increases, the curve gradually bends over, becoming finally almost parallel to the

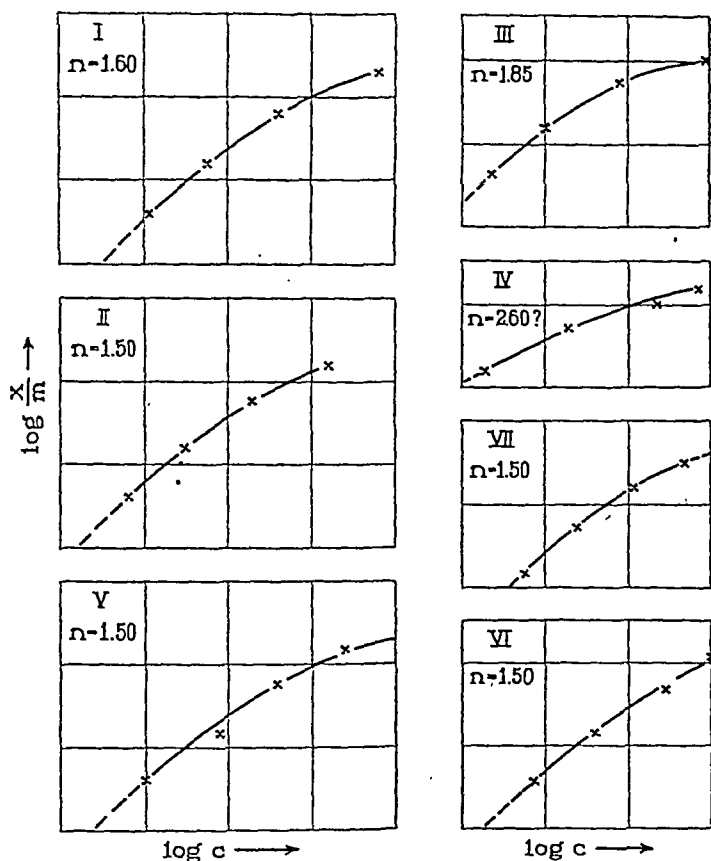


FIG. 12. Deviation of complement fixation from the adsorption isotherm of Freundlich.

$$\log \frac{x}{m} = \left(\frac{1}{n} \times \log c \right) + k'$$

c axis. The complement fixation reaction shows the same deviation from the Freundlich equation (Fig. 12).

D. Summary.

- 1) Since complement fixation has a very low temperature coefficient;
- 2) since its velocity does not conform to that of a bimolecular reaction,

but resembles that of adsorption reactions in general; 3) since the quantitative relationships between complement and fixing agent are not stoichiometric, but conform to those encountered in all adsorption reactions, even though as yet unexplained; and 4) since the reaction obviously takes place at the surface of a heterogeneous phase, we may characterize it as an adsorption process.

The experiments already presented show clearly that all types of complement fixation, whether by the immune globulin suspension, sensitized cells, or agglutinated bacteria, are fundamentally the same

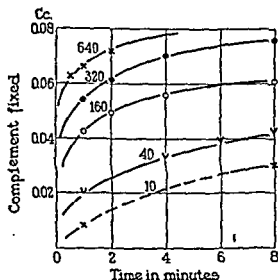


FIG. 13. Effect of immune serum upon complement fixation by agglutinated bacteria. Numbers on the curves indicate relative quantity of immune serum used.

reaction. The adsorbate is always, of course, "complement;" it remains to characterize the adsorbent.

In the precipitation reaction the adsorbent is the precipitate itself (7), (9), (10), (11). The question is, what is the physical basis for the complement fixing property of the antigenic cell after it has been incubated with immune serum?

The experiment summarized in Fig. 13 proves that the complement fixation obtained with bacteria (and presumably¹ red cells) is due entirely to some alteration in the cell surface caused by the immune serum, which is proportional to the degree of sensitization. The most logical hypothesis is that during sensitization there is an aggregation

¹ This has since been shown to obtain for red cells also (13).

of immune-serum globulin upon the surface of the cells, identical with that formed during the precipitation reaction: and that complement fixation in *all* the immune reactions is due to an adsorption by these aggregates.²

The following data already in the literature prove the formation of just such a surface film of immune serum protein during cellular sensitization.

III.

Aggregation of Immune Serum Protein as a Common Factor in all the In Vitro Immune Reactions.

A. Precipitation.

Welsh and Chapman (28), (29), showed that the precipitate which is formed by the interaction of, *e.g.*, sheep serum with a rabbit anti-serum consists almost entirely of denatured immune-serum globulin, with a relatively small quantity of antigenic protein.

B. Bacterial Agglutination.

1. Agglutinated bacteria, washed free of excess serum, will cause anaphylaxis when injected into an animal previously sensitized to the species of serum used (6).

2. Normal bacteria have a negative charge, reversed only by trivalent cations (24), or at a very acid reaction (pH 3.5 (23)). But as the degree of sensitization is increased, their cataphoretic isoelectric point gradually approaches pH 4.7 ((23) horse serum), and, according to Shibley, becomes identical with that of particles of heat-denatured serum globulin ((25) rabbit serum). A phenomenon, noted long before these recent findings, is a direct corollary: specific agglutination is enhanced by cations at serum reaction (when the change in the organisms is negative), and by anions in acid reaction (when the change in the organisms, *determined by the serum globulin surface* is positive) (3), (19), (26).

3. Normal bacteria agglutinate in acid reaction, the optimum range being, to a certain extent, characteristic for each species. When

² This aggregation of immune-serum protein upon the surface of the *individual* cell is not to be confused with the subsequent agglutination of many cells.

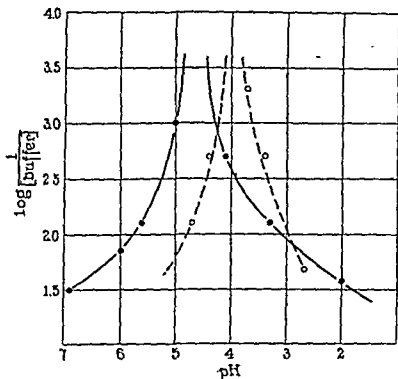


FIG. 14. Effect of pH upon the agglutination of sensitized and normal pneumococci by electrolyte.

———— = Sensitized.
 - - - - - = Normal.

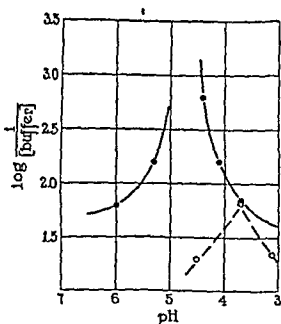


FIG. 15.

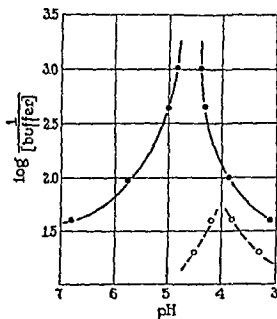


FIG. 16.

FIG. 15. The effect of pH upon the agglutination of sensitized (————) and normal (-----) *B. typhosus* by electrolyte.

FIG. 16. The effect of pH upon the agglutination of *B. pertussis* by electrolyte.

———— = Agglutinated with immune serum.
 - - - - - = Normal.

sensitized, however, all organisms have an optimum agglutination at the same pH as particles of denatured serum globulin (Figs. 14, 15, 16).

Protocol 3.—Suspensions of each of the three organisms named below are incubated with approximately twenty agglutinating units of immune serum, washed 3 times in 20 cc. and resuspended to 50 times the volume of the sedimented cells in H_2O . To 0.2 cc. of each suspension are added 0.8 cc. of varying dilutions of acetate buffers, and agglutination read after 2 hours at room temperature.

In the following figures, the abscissae are the pH of the buffer when N/10 with respect to NaAc; the ordinates represent the minimal concentration of buffer³ necessary to cause complete agglutination. It should be noted that the peaks of the curves correspond to very high dilutions of buffer, at which the pH can only be very roughly approximate. As determined colorimetrically, this correction increases steadily with dilution, and is as much as 0.6 pH at the peaks of the curves (buffer N/4000).

It is seen that immune serum makes the organisms much more susceptible to agglutination by electrolytes, and that there is a sharply defined optimum reaction for agglutination, *the same for all three organisms*, at about pH 5.0 (making the approximate correction).

C. Red Cell Sensitization.

1. Sensitized red cells, when injected into an experimental animal, cause the formation of precipitating and complement-fixing antibodies *against the immune-serum* as well as against the antigenic cell. The only possible explanation is that immune serum protein adheres to the surface of the cell during sensitization, normal sera not causing the same phenomenon.

2. If, to a suspension of sensitized cells, there is added an anti-serum which precipitates the immune serum used, there is an increased agglutination and a greater susceptibility to hemolysis, *i.e.*, the secondary precipitation upon the cell surface, which could only be of protein, functioned exactly like an increase of amboceptor serum (1), (14), (15).

3. The maximum binding of amboceptor by the red cell, as well as the minimum subsequent dissociation (in saccharose) is at pH 5.3, the isoelectric point of serum globulin, where it is most labile, and most readily thrown out of solution (8), (18).

4. The optimum range for the agglutination of sensitized cells is around pH 5.3 (7).

³ (NaAc) is taken as (buffer), the ionization of the HAc being negligible in comparison.

Conclusion.

Knowing that the washed suspension of denatured immune-serum globulins, formed during the precipitation reaction fixes complement powerfully; and that a similar aggregation of immune serum globulin upon the surface of the antigenic cell accompanies bacterial agglutination and red cell sensitization, we may conclude that it is this surface film of protein which determines complement fixation by adsorbing the labile complement adsorbate.

IV.

The Nature of the Attractive Force Between Complement and the Adsorbing Aggregate.

There is one peculiarity of the complement fixation reaction which is not explained by the concept just outlined. Whatever the forces involved, adsorption is by definition a surface reaction. How are we to explain the fact that the precipitate formed in an immune reaction has at least 20 times as great an avidity for complement as a suspension of, *e.g.*, Kaolin, or normal bacteria, or carbon particles of approximately the same microscopic size as the elements of the precipitate? Why should heating this globulin suspension at 90°C. for 30 minutes destroy its fixing properties, so that it becomes as inefficient an adsorbent as the particles just cited? Why does a suspension of heat-denatured normal serum globulin not possess the same avidity for complement? (Fig. 17.)

Protocol 4.—Each of the suspensions listed in Fig. 17 is made up to 15 times the volume of the sedimented particles in NaCl N/7. To X cc. of the suspension are added 0.8 cc. of 1/10 complement and NaCl to 2 cc., and residual complement determined after 2 hours at room temperature.

These questions can only be answered when we know more concerning the essential nature of adsorption reactions in general. If adsorption is determined solely by the *physical state* of the *surface*, irrespective of its chemical character, then we may conceive of the precipitate formed in the immune reaction as possessing, *e.g.*, a sub-microscopic honeycomb-structure allowing for an enormous increase in surface and ability to adsorb: heat would destroy this "inner surface" so that the particles resembled Kaolin, normal bacteria, etc., in their surface (adsorption) properties.

The recent tendency, however, is to regard adsorption as due to the usual chemical forces of attraction operating at the surface of a heterogeneous phase. So considered, some thermolabile substance would be present in the antigen-antibody complex with a specific chemical affinity for complement. Although it is difficult to explain the adsorption of complement, however weak, by such an inert substance as Kaolin on this basis, the question must be left open for further investigation.

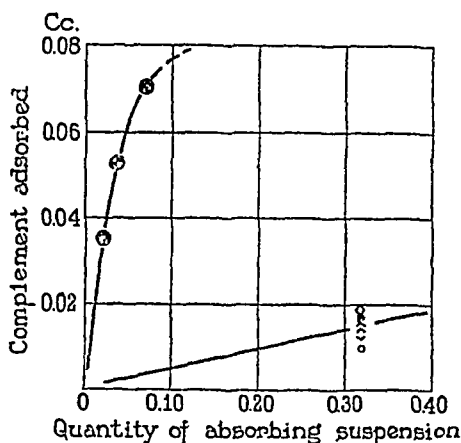


FIG. 17. The adsorptive affinity properties of various suspensions for complement.

- Suspension of specific precipitate. (Sheep *vs.* antiserum.)
- ◇ Specific precipitate heated at 90° ten minutes.
- Heat Denatured egg albumen.
- × Heat Denatured serum globulin.
- △ Heat Denatured serum albumen.
- ▽ Kaolin.
- Normal *killed* bacteria.

SUMMARY.

1. Complement fixation is obtained in every antigen-antibody reaction involving the presence or formation of a heterogeneous phase (red cells, bacteria, precipitate).

2. The physical constants of fixation (temperature coefficient, velocity, quantitative relationships between the reactants) are those

commonly associated with adsorption processes, and are the same in the three types of fixation studied.

3. All the *in vitro* immune reactions involve an aggregation of immune-serum globulins upon the surface of the antigen. It has been shown that the "fixation" of complement is an adsorption by the aggregates so formed; whether these aggregates are visible as a flocculent precipitate (e.g., sheep serum vs. anti-serum) or concentrated as a surface film on a cellular antigen (sensitized cells; agglutinated bacteria), the reaction is fundamentally the same.

4. As yet, it is unknown whether this adsorption is determined by the physical state of the precipitate, and thus, differs only quantitatively from that by Kaolin, charcoal, normal bacteria, heat-denatured proteins, etc.; or whether the comparatively enormous avidity of these aggregates for complement is due to a specific chemical affinity.

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MECHANISM OF HEMOLYSIS BY COMPLEMENT.

I. COMPLEMENT FIXATION AS AN ESSENTIAL PRELIMINARY TO HEMOLYSIS.

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Despite an enormous literature which has accumulated since the discovery of the bactericidal, bacteriolytic, and hemolytic properties of fresh serum, the terms alexin and complement still denote unexplained properties of serum rather than a chemical entity. The hemolysis of sensitized cells, as the most clearly defined of these immunologic properties, has been that most studied, and many theories have been suggested as to its mechanism. The analogy drawn by Noguchi (16) between complement and certain hemolytic lipoids has been found by Liefmann and Cohn (10) to be unwarranted. Dick (3), finding increased amino-acids after hemolysis, was inclined to identify complement as a proteolytic ferment; Olsen and Goette find a suggestive analogy between complement and serum lipase (18); while, more recently, it has been interpreted as an intracellular catalyst allowing the release of hemoglobin by the amboceptor ferment (9). The statement, which we have since found to be erroneous, that complement is not consumed during hemolysis (1), (11), seemed to support the theory of its enzymatic (catalytic) action. At best, the evidence is inconclusive, and the rôle of the preliminary sensitization with immune serum remains unexplained.

I.

Complement Fixation by Sensitized Cells.

It has already been shown (5) that sensitized cells "fix" complement, and that this fixation is exactly similar to that by agglutinated bacteria, or by the precipitate formed when, *e.g.*, sheep serum is added

to a rabbit anti-sheep serum. Because this fixation is obviously a surface reaction, with a very low temperature coefficient, and because

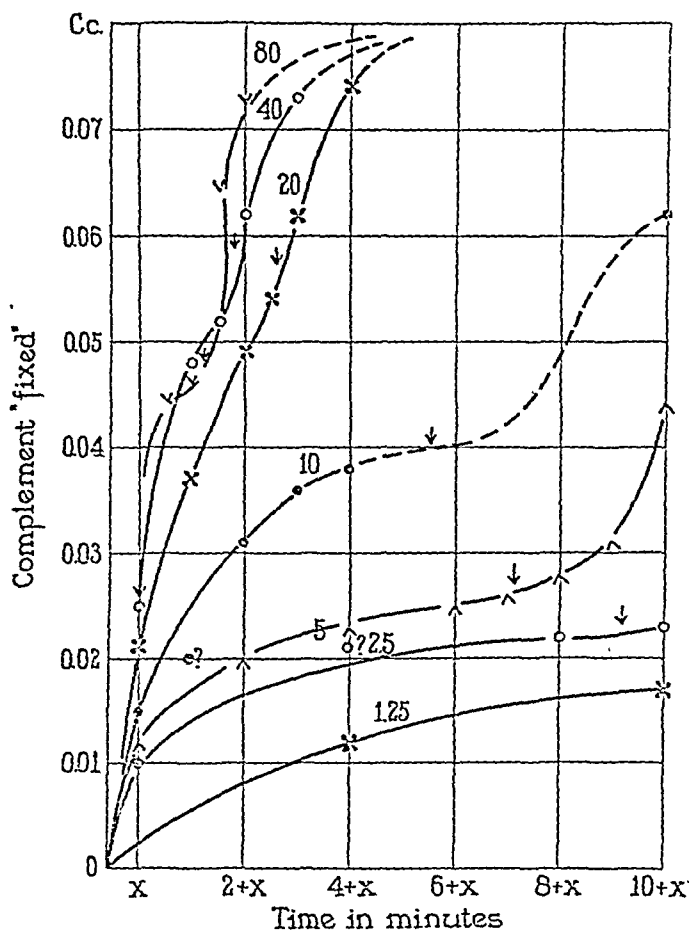


FIG. 1. Effect of degree of sensitization upon complement adsorption by sensitized cells.

To 3.5 cc. of a washed 10 per cent suspension of cells are added immune serum and NaCl $\text{N}/7$ to a total volume of 4 cc. The numbers on the curves are 1000 times the volume of immune serum used in each experiment. After 2 hours at room temperature, 0.4 cc. of the sensitized cell suspension is added to each of 10 tubes and the supernatant fluid removed by *slow* centrifugation. 0.08 cc. complement in 2 cc. NaCl $\text{N}/7$ is added to the sedimented cells at X minutes before centrifugation. This second centrifugation is performed at high speed, for exactly 1 minute, the clear supernatant carefully decanted into a tube containing 0.1 cc. of a standard test suspension, and the residual complement determined by a method already described (4) from the time required for complete hemolysis.

the quantitative relationships conform with reasonable accuracy to the empirical adsorption isotherm of Freundlich, it has been characterized as an adsorption process. The adsorbate is, of course, the labile substance in fresh serum called complement; and it was suggested that the adsorbent is *the same in all the in vitro immune reactions*: the immune-serum protein which adheres on the surface of the antigen during sensitization, agglutination, or precipitation. In the latter, it is visible as a precipitate consisting almost entirely of immune-

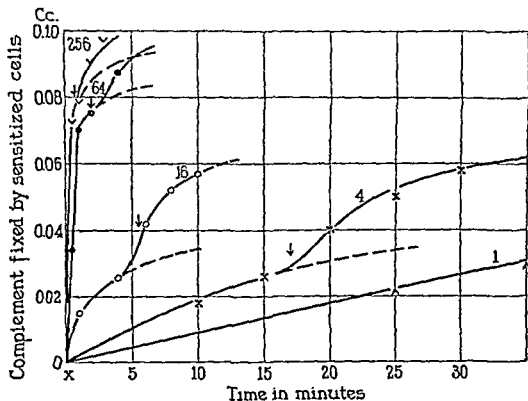


FIG. 2. Effect of degree of sensitization upon complement fixation.

serum globulins, but containing also antigenic protein. In the former it can be demonstrated immunologically, and by changes in the surface properties of the antigenic cell (cataphoresis; agglutination; effect of electrolytes).

The following experiment indicates that this hypothesis is fundamentally correct: using a constant quantity of cells, the velocity and degree of complement fixation is seen to increase enormously with the degree of sensitization, *i.e.*, with the amount of immune serum protein taken up by the cell¹ (Figs. 1 and 2).

¹ Whether or not the immune body is protein, it is always associated with the serum globulin. Increased sensitization results, not only in greater fixation of

It remains to show that this fixation is an essential preliminary to hemolysis; that complement is entirely inactive until it has been so adsorbed by the sensitized cell.

II.

The Adsorption of Complement by the Sensitized Cell as a Determining Factor in Hemolysis.

By definition, complement fixation is the removal (adsorption) from fresh serum of a substance (or substances) essential in immune hemolysis. Since there is always a fixation of complement by the sensitized cell *before* hemolysis (5), it is logical to assume that such a fixation is an essential feature of the reaction. The proof for this assumption is given by the following experiments.

(a) In Figs. 1 and 2 the arrows indicate the approximate time at which hemolysis began. There is obviously some significant relationship between the velocity of fixation and the velocity of hemolysis. Although the nature of the experiment does not justify a quantitative treatment, it is seen that the areas under all the curves from time 0 to the time of beginning hemolysis is *approximately* a constant, *i.e.*, the velocity of the hemolysis reaction at any moment is proportional to the total complement already adsorbed.² The fact that the same result is obtained when we vary, not the degree of sensitization,

complement, but in a more and more pronounced tendency for the cell to assume the surface characteristics of a particle of denatured globulin.

² Let $C_A = f(t)$ = Complement adsorbed by cell

$\frac{dh}{dt}$ = Velocity of the complement-cell reaction, and

H = Amount of this reaction required for hemolysis, obviously
a constant for any one suspension

t_h = Time required for hemolysis

Then $\int_0^{t_h} C_A dt = k = KH = K \int_0^{t_h} \frac{dh}{dt}$ in all the curves.

And $\frac{dh}{dt} = KC_A$: the velocity of hemolysis at any time is proportional to the total complement already adsorbed. This statement is an approximation only of the true relationships, and becomes quite inaccurate at high concentrations of complement. The kinetics of hemolysis will be discussed in a later paper.

but the amount of complement (Fig. 3) indicates a true causal relationship.

(b) The marked influence of electrolyte concentration and the reaction of the solution upon hemolysis is due to the concomitant inhibition of complement fixation.

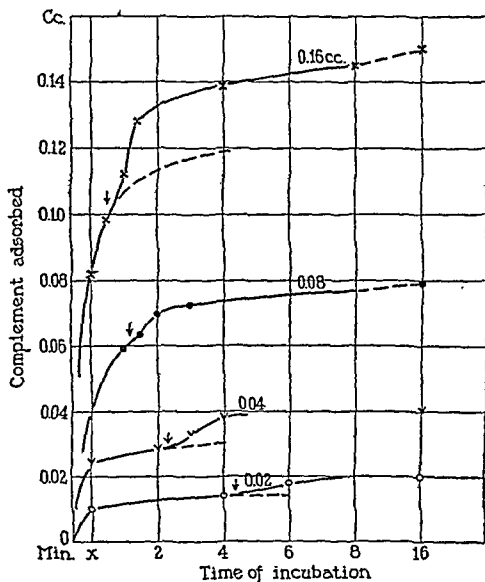
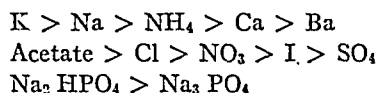


FIG. 3. Effect of (complement) upon velocity and degree of complement adsorption.

One of the earliest observations concerning complement was that it is destroyed by dilution with glucose or water, and that hemolysis does not occur in the absence of electrolytes (7), (13), (19). Manwaring (12) noted the inhibitory effect of electrolytes in excess, and suggested

as its cause the formation of a loosely bound salt-complement compound, from which active complement could be liberated by precipitation of the salt. Noguchi (17) commented on the strong inhibition by salts of Ca or Ba. More recently Wright and MacCallum (23) have tested the effects of many salts in hemolysis, and have arranged the ions in the order of their inhibitory activity in a series analogous to the Hofmeister ion series. Thus



No attempt was made to preserve a constant pH, and as will be seen, this is essential for a proper comparison of the ion effects.

It is also known that the optimum reaction for hemolysis lies at about pH 7.0 to 7.5 (14), and that complement is irreversibly inactivated by acids and alkalis (6). More recently, Brooks (2) has found the destruction to begin at pH 5.3. This is apparently at variance with the previous observation of Michaelis and Skwirsky (14) that if sensitized cells are incubated with complement at pH ± 5.6 (phosphate buffers 1/16) hemolysis does not occur; but if the optimum reaction is subsequently restored by adding alkaline phosphates, the cells are hemolyzed, *i.e.*, the complement was not irreversibly inactivated in acid reaction, but inhibited.

Obviously, the facts require re-investigation. Of interest, however, are not the data so obtained, but their interpretation. For *it will be shown that these changes in the efficiency of hemolysis are due to variations in the efficiency of complement adsorption*. The degree of hemolysis is determined solely by the amount of complement adsorbed, and bears no relation to the total quantity added. And failing adsorption, multiple units of active complement have no effect upon sensitized cells.

1. Methods.

(a) *Hemolysis*.—The rabbit anti-sheep cell system was used throughout. Large quantities of sensitized cells were prepared as follows: To a known volume of sheep's blood were added 10 units of hemolytic serum and several volumes of 0.14 N NaCl (0.85 per cent). After 1 hour's incubation at 37°C., the cells were sedimented by slow

centrifugation, washed once in 20 to 30 volumes of saline and re-suspended to 10 times the original volume of blood, making a stock 10 per cent suspension of sensitized cells (as compared to whole blood).

In order to secure comparable results, a standard method must be used, in which all variables save that studied are kept constant.

Volume.—All tubes are made up to total volume of 2 cc.

Cells.—0.5 cc. of the 10 per cent stock suspension of sensitized cells.

Diluents.—Both the 0.14 N NaCl and 5 per cent glucose used for dilution were brought to pH 7.4.

Incubation.—1 hour at 37°C., followed by at least 2 hours in ice box, or 1 hour at room temperature.

Reading.—

0 = No observable hemolysis.

± = Doubtful.

1 = Distinct partial hemolysis.

2 = Approximately half of cells hemolyzed.

3 = Distinct residual opacity of non-hemolyzed cells.

4 = Complete hemolysis.

The hemolytic unit of complement is arbitrarily defined as the minimal quantity which will completely hemolyze 0.5 cc. of sensitized cells at pH 7.4 in 1/7 N NaCl under the above conditions of time and temperature.

(b) *Complement Fixation as Described in a Preceding Paper* (5).—As in the case of the hemolysis tests a uniform volume pH, tonicity, and time of incubation must be rigidly adhered to for comparable results.

(c) *Electrolytes.*—

NaCl	Na ₂ HPO ₄	CaCl ₂
KNO ₃	Na ₂ SO ₄	BaCl ₂
LiNO ₃	Na ₂ Citrate ³	MgCl ₂
KCNS	Na ₂ Tartrate	MgSO ₄
LiCl	Na ₂ Succinate	
	K ₂ Oxalate ³	

³ Neither K₂ oxalate nor Na₂ citrate could be used. The former, in concentration > 0.2 N, causes a slow hemolysis accompanied by a change in tint, in the absence of complement; the anti-complementary action of the latter is well known, and, as yet, unexplained.

All the salts were prepared in 1 N solution and brought to pH 7.4 by addition of HCl (NaOH) before making the final dilution.

(d) *Hydrogen Ion Concentration*.—This was determined colorimetrically, and checked occasionally by hydrogen electrode. The figures as given are accurate only to 0.1 pH. The shift caused by the addition of sensitized cells, significant even in strongly buffered solutions, was determined by centrifuging out the cells after sufficient time had been allowed for equilibrium to become established.

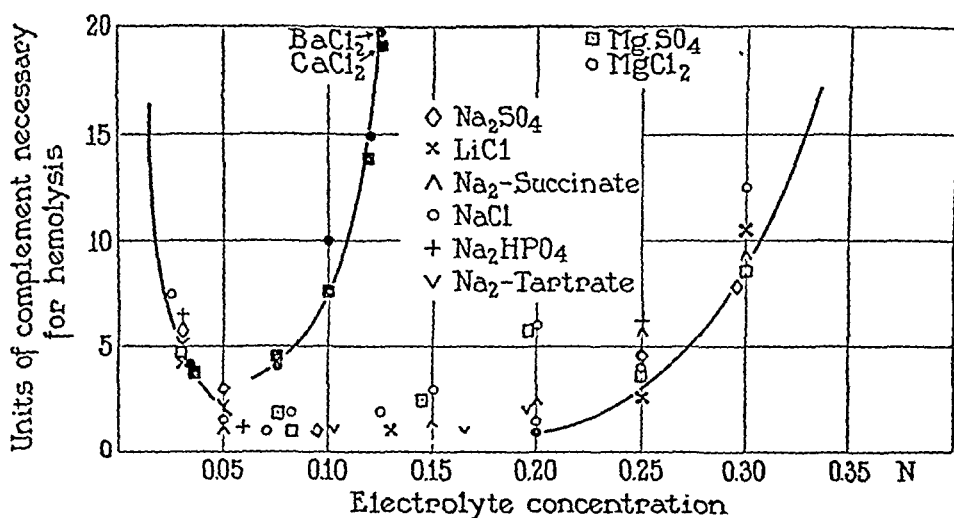


FIG. 4. Effect of electrolyte concentration upon hemolysis by complement.

2. The Effect of Electrolytes.

To a series of tubes containing increasing quantities of electrolytes were added 0.5 cc. of sensitized cells, washed and suspended in 5 per cent glucose, and 5 per cent glucose to a total volume of 2 cc. The minimal quantity of complement necessary for complete hemolysis was then determined for each concentration.⁴

⁴ These experiments were carried out before there was available a rapid method of complement titration. That used here is cumbersome and relatively less precise than either the method of Brooks (2) or that used in experiments already described (4).

Table I gives the data for NaCl. To simplify comparison, the results obtained with the other salts are combined in Fig. 4, in which abscissae represent electrolyte concentration and ordinates the number of complement units necessary for hemolysis.

By varying the electrolyte concentration, three distinct effects are produced upon the hemolytic activity of complement. Below a concentration of 0.025 to 0.03 N, even 20 units of complement fail to cause hemolysis, provided of course, the electrolyte in complement is

TABLE I.
Influence of [NaCl] upon Hemolysis of Sensitized Cells by Complement.

NaCl N/1, cc..... H ₂ O, cc..... Glucose 5 per cent, cc. Sensitized cells in glucose 5 per cent. Final [NaCl] N.....	ISOTONIC								HYPERTONIC			
	1.5	0.025 0.15	0.05 0.3	0.1 0.6	0.15 0.9	0.2 1.2	0.25 1.25	0.3 1.2	0.4 1.1	0.5 1.0	0.6 0.9	0.8 0.7
	0.5	0.5 0.012	0.5 0.025	0.5 0.05	0.5 0.07	0.5 0.1	0.5 0.12	0.5 0.15	0.5 0.2	0.5 0.25	0.5 0.3	0.5 0.4
Complement												
cc. units												
0.015 ½	0	0	±	3	4—	4—	4—	4—	2	0	0	0
0.02 1	0	0	1	4—	4	4	4	4	3	1	0	0
0.025 1½	0	0	1	4					4—	1	0	0
0.03 1½	0	1	2	4					4	2	0	0
0.04 2	1	2	2							3	1	0
0.05 2½	2	0?	2							3	2	0
0.08 4	2	2	3							4	2	0
0.1 5	2	2	4—								4—	1
0.2 10	2	4—	4								4—	2
0.4 20	2	4	4								4	2
Minimal units complement necessary for hemolysis	>20	10-20	5-10	1-2	1	1	1	1	1½	4	10-20	>20

included in the calculation of the final salt concentration. There follows a zone of optimal concentration, whose upper limit for monovalent salts seems to be serum tonicity. Beyond this, increasing electrolyte concentration necessitates more and more complement, until, in 0.4 N NaCl the inhibition is absolute and even 20 units of complement fail to cause hemolysis.⁵

⁵ The puzzling observation of Muir and Browning (15) that hypertonic salt allows complement to pass through a Berkefeld filter, by which it is usually re-

Neither of these two zones of inhibition is due to a destruction of complement. From hypertonic solutions, active complement may be recovered quantitatively by making the proper dilution (Table II). It is true that in hypotonic solution there is an irreversible inactivation of complement function. But in the presence of sensitized red cells this destruction is only partial. Here also, sufficient complement

TABLE II.

Survival of Complement in Hypertonic Solution.

To 0.5 cc. complement are added 0.5 cc. of N/1 electrolyte. After 1 hour at 37°C., the minimal hemolytic quantity of each mixture is determined, using sensitized cells in glucose to avoid hypertonicity.

Mixture of complement and electrolyte	NaCl	LiCl	BaCl ₂	Control (NaCl N/1)
cc.				
0.02	2	3	2	3
0.03	4—	4—	3	4—
0.04	4	4	4	4
0.05	4	4	4	4

remains to completely hemolyze the cells, were it not for an as yet unexplained inhibiting factor; for when the proper tonicity is restored, hemolysis occurs (Table III).

But the most striking feature of the curves plotted in Fig. 4 is the influence of cation valence upon the second zone of inhibition, becoming even more manifest if we prevent the partial hemolysis caused by Ca and Mg salts (Fig. 5). The valence of the anion seems to play no rôle—but with increasing cation valence there is an astonishingly greater inhibitory action. The zone of optimal concentration is narrowed, and the succeeding inhibition much more pronounced. At

tained, is explained by these findings. Complement fails to pass the filter, not because of its molecular dimensions, but because it is adsorbed by the finely particulate material of which the filter is made. Hypertonic salt inhibiting such adsorption, the complement passes.

Similarly, the fact that after repeated passages of fresh serum, a filter finally becomes permeable to complement, is due to the saturation of the filter adsorbent with the complement adsorbate.

TABLE III.

Inhibition of Hemolysis in Hypotonic Solution not Entirely Due to Destruction of Complement.

Complement	Inhibition of hemolysis		Destruction in absence of cells	Control
	(a)	(b)		
	Cells 0.5 cc. Glucose 1.3 cc. Complement 37° 1 hour	NaCl N/1 ~0.2 cc. added after incubation	Glucose 1.3 cc. Complement After 1 hour at 37° add Cells 0.5 cc. NaCl N/1 0.2 cc.	Glucose 1.3 cc. Cells 0.5 cc. NaCl N/1 0.2 cc.
cc.	units			
0.015	2	0	2	3
0.02	1	0	3	4
0.04	2	0	4	4
0.05	2½	0	4	4

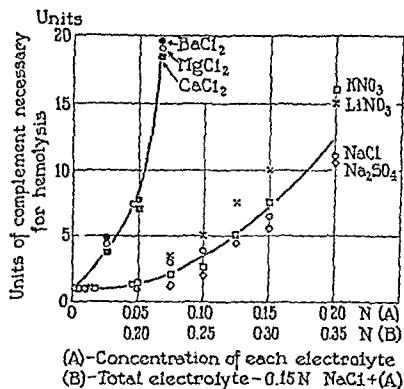


FIG. 5. Effect of electrolytes upon hemolysis (non-specific hemolysis by salts prevented in hypertonic solution).

To 0.5 cc. of sensitized cells in 0.15 N NaCl are added X cc. of $N/1$ electrolytes, each containing, in addition to the individual salt, 0.85 per cent NaCl (0.15 N). 0.15 N NaCl is added to a total volume of 2 cc., and the minimal hemolytic quantity of complement determined at each concentration.

The hemolysis caused by the salts themselves is almost completely inhibited in such hypertonic solutions.

TABLE IV.

Effect of Electrolyte Concentration upon Fixation of Complement by an Immune Globulin Suspension.

Complement is incubated with globulin at varying concentrations of NaCl. After 1 hour at 37°, to Y cc. of each tube (figures in left hand column), are added 0.5 cc. sensitized cells, and NaCl N/7 to a total volume of 2 cc. The figures in the body of the table represent degrees of hemolysis.

Globulin suspension, cc.	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Complement, cc.		0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
NaCl N/7, cc.		0.25	0.25	0.5	1.5	1.45	1.4	1.35	1.3	1.25
NaCl N/1, cc.						0.05	0.1	0.15	0.2	0.25
Glucose 5 per cent, cc.	1.7	1.5	1.25	1.0						
Final NaCl normality	0.014	0.035	0.055	0.07	0.14	0.16	0.18	0.21	0.23	0.25
cc.										
0.1	±	3	1	0	0	±	1	2	3	4-
0.2	3	4	4	0	2	3	3	3	4-	4
0.3	4	4	4	0	3	4-	4	4	4	4
0.5			4	2	4	4				
0.6				2						
1.0				3						
Total units complement remaining free.....	7	10	10	<2	4	4-6	7	7	7-10	10-20
cc.										
Control without { 0.1		3	4	4	4	4				
globulin { 0.2	3	4	4	4	4					
{ 0.3	4	4	4							
Units complement remaining in control....	7	10	20	20	20	20				
Units complement adsorbed by globulin....	0	0	10	>18	16	14-16	13	13	10-13	0-10
Per cent complement adsorbed.....	0	0	50	>90	80	70-80	65	65	50-65	0-50
Amount of complement from which one unit would be adsorbed....	>20	>20	2	1+	1+	1+	1+	1+	1-2	2-20

0.07 N BaCl_2 it required 20 times as much complement to cause hemolysis as it does at 0.07 N NaCl . The limiting concentration for hemolysis where even 20 units fail to have any effect is 0.35 M KNO_3 , but only 0.07 M CaCl_2 .

As compared with such a pronounced valence effect, the differences between individual ions of the same valence are negligible, and do not justify their arrangement into an ionic series.

If we now carry out a similar series of experiments to determine the effects of electrolytes upon complement fixation, we obtain a series of

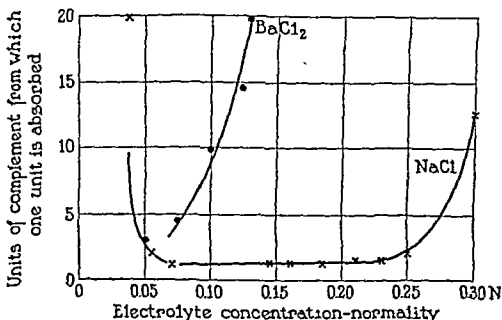


FIG. 6. Effect of electrolytes upon complement fixation.

With the more accurate velocity method of complement titration, one obtains a somewhat more sharply defined optimum zone for fixation. The limiting electrolyte concentrations, however, remain the same.

curves which are, within the limits of experimental error, exact duplicates of the curves for hemolysis. There is the same inhibition in hypotonic solution, the same optimum zone, and the same succeeding inhibition in hypertonic solution, again determined by the cation (Table IV and Fig. 6).

Knowing as we do that sensitized cells adsorb complement, the correlation between adsorption and hemolysis proves that the inhibition of hemolysis with changing tonicity is due to inhibited adsorption—that adsorption is an essential preliminary to hemolysis.

3. The Effect of Hydrogen Ion Concentration.

The effect of changing pH upon hemolysis is summarized in Fig. 7. The irreversible destruction of complement in alkaline reaction is seen to begin at pH 8.5. Its causes are unknown, and need not concern us here. In the acid range, the hemolytic titer of complement begins to decrease at pH 5.9 as compared with 5.3 under the conditions used by Brooks. But it should be noted that sensitized cells in some

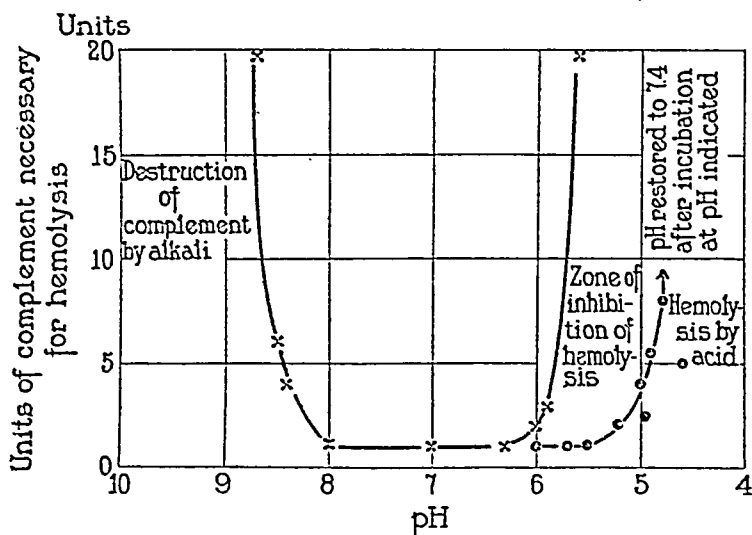


FIG. 7. Effect of hydrogen ion concentration upon hemolysis.

Complement is brought to the desired pH by the addition of NaOH N/7 (HCl). To X cc. of this mixture are added 0.5 cc. of sensitized cells and NaCl N/7 to a total volume of 2 cc. A significant change in reaction occurs when cells are added, and this must be determined for each quantity of complement used.

way prevent the destruction of complement between pH 5.9 to 5.0. When complement is brought to this pH and sensitized cells added at once, hemolysis does not occur, but not because the complement is destroyed, as it would have been in the absence of cells. When the optimum pH is subsequently restored by the addition of NaOH, hemolysis occurs, just as Michaelis and Skwirsky showed. The mechanism of this protective action is intimately related to the problem of midpiece and endpiece. Of interest in the present discussion

TABLE V.

Effect of pH upon Complement Fixation by Immune Globulin Suspension.

20 units of complement (0.4 cc.) are brought to the desired pH by acetate buffer, as indicated in the table, and incubated at 37°C. for 1 hour. To Y cc. of each tube (figures in left hand column), are then added 1), the neutralizing quantity of NaOH, 2), sensitized cells, and 3), NaCl N/7 to make 2 cc. The figures in body of table indicate degrees of hemolysis. The units remaining after incubation obviously = $\frac{2}{\text{minimal hemolytic quantity}}$.

HAc N/7 (in NaCl), cc.....		0.05	0.07	0.1	0.15	0.22	0.3
NaAc N/7, cc.		0.3	0.3	0.3	0.3	0.3	0.3
Complement, cc.	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Globulin in suspension, cc.	0.1	0.1	0.1	0.1	0.1	0.1	0.1
NaCl N/7, cc.	1.5	1.15	1.1	1.1	1.05	1.0	0.9
Final pH	7.4	6.5	6.2	6.0	5.7	5.4	5.1
cc.							
0.05	0	0	0	0	3	2	1
0.1	0	0	0	1	4	4	4
0.2	0	0	2	4	4		
0.5	0	0	2	4			
1.0	0	0	3				
Total units remaining free ..	0	0	<2	10	20	20	20
cc.							
Control without globulin { 0.05	3	3	3	3	3	2	1
{ 0.1	4	4	4	4	4	4	3
{ 0.2	4	4	4	4	4	4	4
Units remaining in control. . .	20	20	20	20	20	20	10*
Per cent complement adsorbed by globulin suspension.....	100	100	>90	50	0	0	0
Amount of complement from which one unit would be adsorbed.	1	1	1+	2	>20	>20	>20

* Beginning destruction of complement by acid reaction.

is the fact that there is a true zone of inhibition at pH 6.0 to 5.0, in which active complement fails to produce hemolysis.

Here also, the puzzling effects upon hemolysis are completely explained by the corresponding changes in complement adsorption by a suspension of globulin particles (and of red cells).

Table V is the protocol illustrating the method used to arrive at the data charted in Fig. 8. The inhibition of complement fixation at pH 5.9 quantitatively parallels and explains the inhibition of hemolysis.

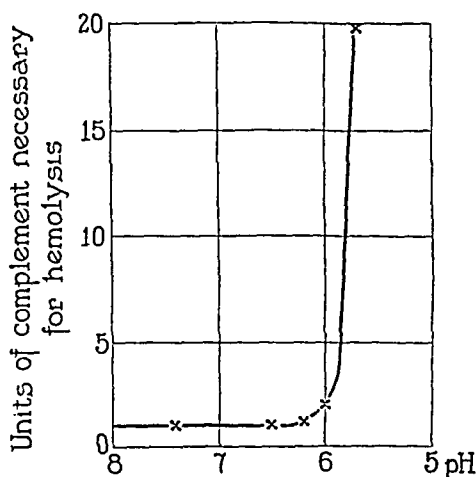


FIG. 8. Effect of pH upon complement fixation by a suspension of denatured immune globulins.

SUMMARY AND DISCUSSION.

1. Sensitization confers upon the red cell the property of adsorbing complement from solution. The submicroscopic film of immune serum protein deposited upon the cell surface during sensitization, and completely analogous to the precipitate formed in a soluble antigen-antibody reaction (*e.g.*, sheep serum vs. rabbit anti-sheep serum) acts as absorbent, the degree of sensitization (size of the film) determining the amount of complement "fixed" (adsorbed).

2. This adsorption of complement by the sensitized cell is an essential preliminary to hemolysis, and when inhibited, even large quantities of demonstrably active complement have no hemolytic action.

The marked influence of electrolytes and of the hydrogen ion concentration upon hemolysis is due primarily to corresponding effects upon the fixation of complement by the sensitized cell. In the case of salts with monovalent cations, complement fixation (and hemolysis) is completely inhibited at any concentration < 0.02 M or > 0.35 M. Electrolytes with bivalent cations are much more inhibitory, and in low as concentration 0.07 M completely prevent fixation (and hemolysis).

The optimal reaction for complement fixation (and hemolysis) is pH 6.5 to 8.0. In slightly more acid range both are *inhibited*. But at a reaction pH 5.3, and in the alkaline range, there is an irreversible inactivation of complement, complete at pH 4.8 and 8.8 respectively.

It is perhaps more than a coincidence that complement fixation, and therefore, hemolysis, are prevented by just those factors which suppress the ionization of serum proteins, and lead to an increased aggregation state. Between a suspension of macroscopically visible particles of euglobulin in distilled water, and a solution in physiological saline, there is certainly a gradual transition, manifested at low electrolyte concentrations by the opacity of the solution. At pH 7.4, globulin would ionize as a Na-salt, an ionization inhibited as the isoelectric point (5.3) is approached, with a coincident greater tendency of the globulin to separate from solution. And the cataphoretic velocity of particles of globulin, as well as all the other properties which are a function of its ionization (viscosity, osmotic pressure, etc.), are suppressed by electrolytes, the degree of suppression being determined by the concentration and valence of the cation (on the alkaline side of the isoelectric point). The analogy with complement fixation is too complete to be dismissed as fortuitous.

3. The fact that the degree of complement "fixation" increases with the degree of sensitization explains one of the most puzzling phenomena in hemolysis,—that immune serum and complement are, to a certain extent, interchangeable, a decrease in either factor being compensated by an increase in the other (8), (20), (22). The explanation is evident from Figs. 1, 2, and 3. The exact quantitative relationships involved will be developed in a later paper. With increasing sensitization there is an enormously more complete and more rapid fixation of complement, and correspondingly more rapid hemolysis, exactly

the effect produced by increasing the quantity of complement instead of amboceptor (Fig. 3). All other variables being constant, the velocity of hemolysis is determined by the amount of complement adsorbed. With more amboceptor, a greater proportion is "fixed" by the cell; with more complement, a smaller proportion, but a larger absolute amount. The result is the same: more complement adsorbed, and a corresponding acceleration of hemolysis.

If this mobilization of complement is the sole function of immune-serum (and there is as yet no reason to assume any other), then the accepted terminology, in which amboceptor, immune body, and hemolysin are used synonymously, is erroneous. The immune body would function only as an "amboceptor," mobilizing the effective hemolysin, complement, upon the surface of the cell.

Nothing has been said of the multiple components into which complement may be split. *A priori*, it would be expected that the adsorption demonstrated is of the so called midpiece fraction.

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THE PENETRATION OF LUMINOUS BACTERIA BY THE AMMONIUM SALTS OF THE LOWER FATTY ACIDS.

PART I. GENERAL OUTLINE OF THE PROBLEM, AND THE EFFECTS OF STRONG ACIDS AND ALKALIES.

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In the experiments of Jacobs¹ on hemolysis by the ammonium salts of the lower fatty acids, hemolysis is used as a measure of penetration. It is concluded that only the molecules of ammonia and of fatty acid, formed by hydrolysis of the salt, are effective in penetrating the cell membrane. These reunite within the cell to form the salt, cytolysis resulting from the increased osmotic pressure.

It was thought that some of these studies might be repeated, using luminous bacteria as the experimental material. The problem as originally conceived was a study of the rates of cytolysis of luminous bacteria by the ammonium salts of formic, acetic, propionic, butyric, and valeric acids, using solutions of all pH values at which measurements of the time of cytolysis could be made. Cytolysis would be detected by the disappearance of the light and would indicate penetration. As the work progressed it became apparent that disappearance of the light did not always indicate cytolysis but under the conditions of the experiments did indicate penetration, and numerous modifications of the original procedure were suggested. Before attempting

* The experiments described herein were carried out in the physiological laboratory of Princeton University and in the laboratory of the United States Bureau of Fisheries. I wish to acknowledge my indebtedness to Mr. Henry O'Malley, Commissioner of Fisheries, and to Mr. Elmer Higgins, Director of the Bureau of Fisheries Laboratory at Woods Hole, for placing the facilities of the laboratory at my disposal. I am also deeply indebted to Prof. E. Newton Harvey of Princeton University, under whose direction this work was done, for constant advice and guidance.

¹ Jacobs, M. H., Harvey lectures, 1927, xxii, 146.

analysis of the data obtained with the ammonium salts it was therefore necessary to answer a number of questions bearing on these points, as follows:

Are luminous bacteria cytolyzed as erythrocytes are by water, hypotonic non-penetrating salt solutions, and by penetrating solutions of all concentrations? Is disappearance of the light coincident with cytolysis? May disappearance of the light be used as a criterion of cell penetration? May the presence of the ammonium salts within the cell be responsible for the extinction of the light, independently of osmotic effects? Are the bacteria injured by the activity of hydrogen and hydroxyl ions in the range of pH values covered by the ammonium salts? Is the point of injury the same, whether hydrogen or hydroxyl ion activity is exerted on the outer surface of the cell membrane or on the protoplasm itself? May salt injury and acid and base injury be separated from the osmotic effects of the ammonium salts?

These questions will be considered under subsequent headings.

I.

Experimental Methods.

The organism studied was *Bacillus Fischeri* (Beijerinck, Migula), a marine form isolated from herring by C. S. Shoup at Woods Hole during the summer of 1927 and since maintained in pure culture in my laboratory. The bacteria were grown in Petri dishes, using the calcium carbonate buffered media described in a previous paper.² The cultures were used when approximately 24 hours old, the bacteria being brushed from the surface of the medium and collected in M/2 NaCl.

A single drop of bacteria suspended in M/2 NaCl was added to 10 cc. of solution, the tube vigorously shaken, and watched until the light disappeared. In some early experiments the attempt was made to use a standard low intensity of light as the end-point of the reaction. The results thus obtained were so erratic and unreliable that these experiments were discarded and total disappearance of the light taken as the end-point. The eye is sensitive to light of so low an intensity that measurements are difficult, and Reeves³ has shown that this threshold varies from day to day. In order to minimize errors due to this variability as well as to variability in the bacteria, experiments were always run in multiple. No measure-

² Hill, S. E., *Biol. Bull.*, 1928, lv, 143.

³ Reeves, P., *Abridged Scient. Pub. Research Lab. Eastman Kodak Co.*, 1917-18, iii, 13.

ments are recorded except those made after at least 1 hour spent in light of low intensity, and at least 15 minutes in total darkness. The use of a stop watch under such conditions is obviously impossible. The timing unit was therefore a metronome adjusted to beat seconds. The experiments were run in a definite order and the results recorded on a "Sunstrand" adding machine, which possesses a type of keyboard that can be operated in the dark without error. The records were transcribed to sheets at the close of the experiment.

The pH values of the solutions were measured with either the quinhydrone or the glass electrode, and in a few cases calculated values were used, as for NaOH solutions above pH 9.0. In every case, the method of determining pH is given.

It was early found that a single suspension of bacteria could not be kept in good condition for the 4 or 5 hours necessary to run the longer experiments. In the experiments which were run in multiple, a separate culture of bacteria was used for each of the 8 or 9 parts. The suspension was aerated with a stream of air. In each case the first experiment was run from the acid to the alkaline end of the series, the second in reverse order, etc., in order to equalize any differences due to ageing of the suspension, but no definite differences were found. In all cases the average time of disappearance of the light in 8 or 9 experiments was plotted on the vertical axis and the concentration of free acid or alkali, or pH of the solutions on the horizontal axis.

II.

Applicability of Method.

Luminous bacteria are cytolized by water, hypotonic non-penetrating solutions, and by penetrating solutions of all concentrations. This cytolysis differs from that of erythrocytes because of the difference in shape of the cell, and because the bacillus possesses a rigid cell wall which prevents swelling. The mammalian erythrocyte, because of its peculiar shape, can increase considerably in volume without an increase in surface.¹ The surface membrane can then stretch to some extent before any great resistance is offered to osmotic swelling. With the bacillus, great mechanical resistance is at once offered to osmotic entrance of water, and the cell bursts without increasing in size. The evidence on which these statements are made is presented below.

If luminous bacteria are placed in diluted sea water, there is little change in intensity of the light until the sea water decreases to 20 per cent. Solutions from 20 to 6 per cent sea water form a series of decreasing brilliance, and after a lapse of 5 minutes no light is observed in solutions more dilute than 6 per cent sea water. Viewed in day-

light, the solutions were decreasingly turbid with decreasing sea water concentration, a marked difference being observed between the 100 per cent and 6 per cent solutions. On vigorous shaking, foam developed in all of the solutions, but persisted only in those of sea water concentration below 6 per cent. This indicated the bursting of the cell membrane and the liberation in the solutions of foam-producing substances such as the cell proteins. Examined by transmitted light, the bacteria in the solutions of less than 6 per cent sea water were almost impossible to find but were easily demonstrated in the dark field. In solutions of above 6 per cent they were demonstrable also by transmitted light. Mrs. E. B. Harvey kindly measured a number of bacteria for me, finding only a slight increase in size in 20 per cent sea water.

In another experiment carefully measured quantities of bacterial suspension were added to a series of sea water dilutions as before and counts of the bacteria made in the dark field. No significant differences in number were observed even between 2 per cent and 100 per cent sea water. As before, when the solutions were examined by transmitted light it was difficult or impossible to demonstrate the bacteria in the more dilute samples.

The difficulty of demonstration of cytolysed bacteria by transmitted light is thought to be due to the filling of the cell capsule with water, so that light passing through is not appreciably deflected from its course. With dark field illumination the surface only is concerned and the effect is therefore the same whether we are dealing with a normal cell or a cytolysed one.

In distilled water or tap water the light disappeared in about 15 seconds. The presence of low concentrations of NaCl (up to 0.0156 M) delayed the disappearance of the light for a few seconds and in solutions of 0.0625 M and upward the light lasted for over 1 hour although greatly dimmed in 0.0625 M NaCl. The persistence of the light, undimmed, through a considerable range of NaCl dilution and its great dimming shown progressively through several dilutions, followed by sudden disappearance in slightly more dilute solutions, indicate the rupturing of the cell membrane at a critical osmotic pressure, and the presence of different degrees of resistance in the bacterial population. The least resistant were cytolysed by 0.0625 M NaCl and the most

resistant by 0.0156 M NaCl. These differences might be individual variation or a combination of individual variation with age groups in the population. The results of microscopic examination of the NaCl dilutions were in every way similar to those of the sea water dilutions.

Sucrose was almost as efficient as sea water or NaCl in preserving the luminescence, as the light disappeared in solutions of 0.0625 M and below, and exhibited a series of increasing brilliance as the sugar concentration rose. This indicated that the failure of light in diluted sea water was an osmotic phenomenon due to bursting and was not

TABLE I.

Time Required for Complete Disappearance of Light of Luminous Bacteria in Hypotonic Solutions.

No examination was made after 6 hours.

Per cent of sea water		Concentration of NaCl		Concentration of sucrose	
100.0	+	0.5 M	+	1.0 M	+
50.0	+	0.25	+	0.5	+
25.0	+	0.125	+	0.25	6 hrs.
12.5	6 hrs.	0.0625	6 hrs.	0.125	1 hr.
6.0	5 min.	0.0312	5 min.	0.0625	5 min.
3.0	1 "	0.0156	1 "	0.0312	1 "

due to the absence of the customary salts as such. This had been previously observed by Harvey⁴ for an unidentified species of luminous bacteria.

In solutions of ethyl alcohol of all concentrations from molar downward the light disappeared in a few seconds as in pure water, while in presence of sea water or 0.5 M NaCl similar concentrations of alcohol were without effect. This shows that the effect of the alcohol in pure water was caused by its easy penetration, and was not due in any way to narcosis or to an attack on the cell membrane.

In solutions of glycerine from molar downward the time of disappearance of the light was scarcely distinguishable from that in pure water, being perhaps 20 per cent longer. In presence of 0.5 M NaCl

⁴ Harvey, E. Newton, *Biol. Bull.*, 1915, xxix, 308.

these concentrations of glycerine were without effect on the luminescence, showing that glycerine also penetrates these cells with great readiness.

Urea did not penetrate as readily as alcohol and glycerine, the disappearance of the light occurring in *M* urea in about three times the time required in pure water (Table II). As before, addition of 0.5 *M* NaCl prevented the disappearance of the light.

It will therefore be seen that with hypotonic or with freely penetrating non-injurious substances disappearance of the light is undoubtedly coincident with cytolysis. With freely penetrating

TABLE II.

Time of Disappearance of Light in Indicated Concentrations of Urea.

[Urea]	In water	In 0.5 <i>M</i> NaCl
1.0 <i>M</i> .	41 sec.	+8 hrs.
0.8 "	38 "	+8 "
0.6 "	36 "	+8 "
0.4 "	32 "	+8 "
0.2 "	29 "	+8 "
0 "	15 "	+8 "

injurious substances the cell processes may be interfered with and disappearance of the light thus precede cytolysis, or cytolysis may not occur. Since the luminescence of bacteria is intracellular, as has been shown by Harvey⁵ and others, any change in intensity of light must be due to changes within the cell. The disappearance of the light indicates cytolysis, anesthesia, the presence of some destructive substance within the cell, or cessation of function because of unfavorable environmental conditions, especially lack of oxygen.

The light may be reversibly extinguished in several ways, as follows: (a) Increase of temperature above a certain critical value, not yet determined for this species, but above 30°. This point has been determined by several investigators for other species.^{6,7,8} (b)

⁵ Harvey, E. Newton, *The nature of animal light*, Philadelphia, 1919.

⁶ McKenney, R. E. B., *Proc. Biol. Soc. Washington*, 1902, xv, 213.

⁷ Harvey, E. Newton, *Biochem. Bull.*, 1913, ii, 456.

⁸ Morrison, Thos. F., *J. Gen. Physiol.*, 1925, vii, 741.

Decrease of oxygen pressure to a value less than 0.0053 mm. Hg.² (c) Presence of anesthetics in sub-lethal concentrations.⁴ (d) By concentrated non-penetrating salt solutions, as by 2.5 M NaCl, the disappearance of the light in this case being due doubtless to plasmolysis.

In all experiments described in this paper concentrated salt solutions have been avoided, presence of ample quantities of oxygen has been assured, and all experiments were conducted at $19 \pm 1^\circ\text{C}$. The disappearance of the light under the conditions of the experiments must therefore be due to osmotic swelling of the cell or to the entrance of injurious substances. When isotonic solutions are tested the disappearance of the light may be used as a physiological criterion of cell penetration whether the failure of the light is due to osmotic swelling or to injury due to the presence of some foreign substance within the cell. The case of internal injury, distinct from osmosis, may be detected by the addition of 0.5 M NaCl to the solutions, thus preventing cytolysis. In any case, disappearance of the light is an indication of cell penetration.

With non-penetrating substances such as strong acids and bases entrance would probably follow destruction of the cell membrane. Disappearance of the light might follow injury to the membrane and precede entrance of the destructive agent to the cell. Entrance after destruction of the cell membrane can hardly be regarded as penetration, but this case is easily detected by the behavior described in the next section.

and lasted for progressively longer periods of time as the acid mixture was diluted with 0.5 M NaCl. At pH 4.0 the light lasted for over 5 minutes, at pH 4.67 for 20 minutes, and at pH 5.46 for over 4 hours. The nature of the curve for the shorter times is shown in Fig. 1. Since none of the ammonium salts employed were more acid than pH 5.0 and since the times measured with the ammonium salts were in all instances 5 minutes or less, it is obvious that injury due to activity of hydrogen ions in the external solutions may be excluded from consideration. Similar results were obtained with NaOH, a non-penetrating base. With one series the pH values were calculated on the assumption that NaOH is completely dissociated, and in the other the glass electrode was used, corrections being applied from the measurements of pH values of ammonia in water and in 0.5 M NaCl solution. In these two series of experiments, the pH was determined after the experiment, so the glass electrode determinations are probably more accurate than the calculated values, since some carbon dioxide was absorbed from the air and some produced by the bacteria in the course of the experiment. At pH 9.0, the upper pH value for most of the ammonium salts employed, the bacteria were still luminous after 2 hours, and it was only above pH 10.0 that any immediate effects could be observed. Damage due to activity of hydroxyl ions in the external solution may also be disregarded.

An outstanding peculiarity of the behavior of luminous bacteria was noted in both HCl and NaOH, as compared with the free fatty acids and with ammonia. In 0.025 M solution of any fatty acid in 0.5 M NaCl the disappearance of the light was so rapid that the time could not be measured, while in 0.025 M HCl in 0.5 M NaCl the light persisted fully brilliant for approximately 6 seconds, then failed completely in less than 1 second. In 0.025 M ammonia in 0.5 M NaCl the light was immediately dimmed to a very low value and then lasted for some time before being finally extinguished. In 0.025 M NaOH in 0.5 M NaCl the light lasted at full value for about 4 seconds, then disappeared suddenly. This complete resistance for a certain period of time, followed by sudden complete failure, indicates that the protoplasm is protected from the strong non-penetrating HCl or NaOH until the membrane is destroyed, the destruction of the then unprotected protoplasm following almost immediately. The HCl or NaOH

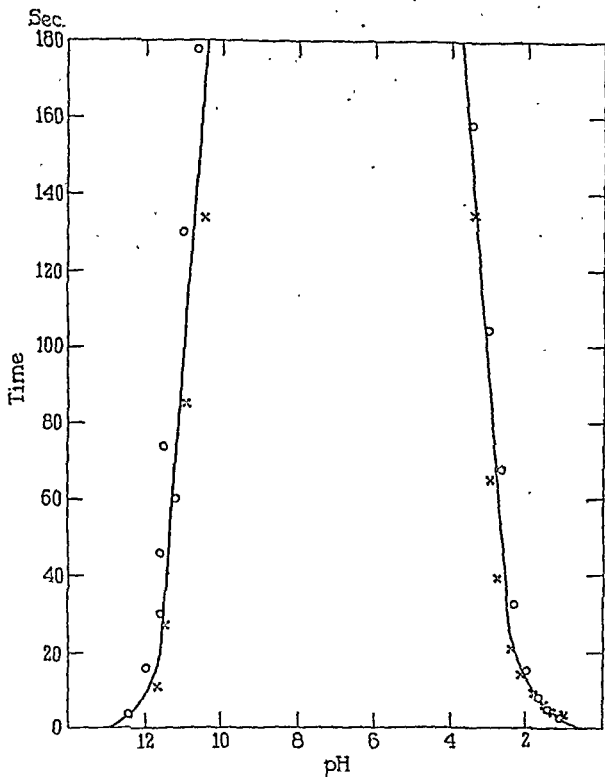


FIG. 1. Time of disappearance of light (in seconds) in solutions containing 0.5 M NaCl plus NaOH or HCl. Each circle is average of 9 experiments, each X of 10. For the circles, pH was determined with the quinhydrone electrode on the acid side, calculated on the alkaline side. For the X's, pH was determined with the glass electrode, with appropriate corrections.

must combine with some substance in the cell membrane, forming a permeable substance, or else simply etch the membrane, before entrance is possible; whereas the ammonia and fatty acid pass freely through without the necessity for first making a path for themselves.

This is similar to the entrance of NaOH into *Paramecia* stained in neutral red, described by Harvey.¹⁰ In 0.002 M NaOH no change in color is seen for 8 or 10 minutes, when there is a sudden change in color of the entire animal. In 0.001 M ammonia a color change from red to yellow begins at the moment the animals are placed in the solution. A similar behavior was observed for *Elodea* leaves stained in neutral red.

SUMMARY.

It is shown that disappearance of the light of luminous bacteria may be used as a criterion of cell penetration; that luminous bacteria are cytolyzed by water, hypotonic solutions, and by freely penetrating solutions; that luminous bacteria are not injured by hydrogen or hydroxyl ions in the external solutions within the range of pH values employed with the ammonium salts and that therefore disappearance of the light in isotonic solutions of these salts must be due to penetration of the solute; and that there is a characteristic difference between the effects of strong and of weak acids and alkalies on luminous bacteria.

¹⁰ Harvey, E. Newton, *J. Exp. Zool.*, 1911, x, 507.

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